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REMARKS

1. Procedural Matters

A Notice of Appeal was filed on June 5, 2006. However, no appeal brief has yet been filed. Hence, this amendment is governed by 37 CFR 41.33(a) and 1.116.

2. Claim Objections

Claim 9 has been amended to depend from claim 30 rather than cancelled claim 8.

3. Definiteness

Claim 11 has been amended to strike the questioned term "essentially".

4. Prior Art

The Examiner has conceded that claim 11, drawn to Lactococcus lactis subspecies lactis strain DN221 is free of the prior art.

However, claims 6, 7, 9, 10, 27 and 30 stand rejected as anticipated by Hugenholtz or Starrenburg in light of evidence by ATCC catalogue. This rejection is respectfully traversed.

Claim 6 has been amended to put this case into better condition for allowance or appeal.

As admitted by the Examiner in the paragraph bridging pp. 3-4 of the office action, Hugenholtz discloses a Pfl-defective lactic acid bacterium belonging to the genus Leuconostoc. Likewise, as noted in the first full paragraph on page 4, Starrenburg's teachings are directed to the metabolic properties of Leuconostoc bacteria.

In contrast, claim 6 as examined recited five other genera: Lactococcus, Lactobacillus, Pediococcus, Streptococcus, and Bifidobacterium.

The Examiner has previously argued that the claimed

Lactobacillus and Pediococcus (there are three other claimed genera) could be anticipated by Leuconostoc deposits because Leuconostoc bacteria have on occasion been reclassified into the claimed genera, and that in turn these two genera have on occasion been reclassified into the other three genera. We responded that (1) the evidence was consistent with simple mis-identification of the strain by the depositor, (2) reclassification of Leuconostoc into Lactobacillus or Pediococcus after August 22, 1996 is legally irrelevant; (3) Hugenholtz's Leuconostoc genus is still considered distinct; (4) Starrenburg's Leuconostoc sp. 60 is definitely Leuconostoc lactis, not a claimed strain; and (5) the PTO has not made out a prima facie case for identifying Starrenburg's Leuconostoc mesenteroides strain 7-1 as Pediococcus, or for asserting that it satisfies clauses (i)-(v) of claim 6.

The Examiner's reply appears on page 5-6 of the office action. She says that the taxonomic assignment of the lactic acid bacteria can change (ignoring our argument that the ATCC catalogue doesn't prove that this occurred) but that the metabolic pathways of lactic acid bacteria remain the same.

There are really several issues here:

(1) Are there in fact an art-recognized taxonomic distinctions between Leuconostoc and the claimed genera?
(2) If such a distinction exists, would the routine worker in the art discount such distinctions when seeking to make a Pfl-defective lactic acid bacterium, if the bacterium in question had a lactic acid metabolism similar to that of Leuconostoc?
(3) Are the lactic acid metabolisms of the bacteria of the claimed genera sufficiently similar to that of Leuconostoc so that the routine worker in the art would find it obvious to apply the teachings of the Leuconostoc references to those claimed genera?

With regard to issue (1), we don't think that there is any doubt that the art recognizes the existence of a taxonomic

distinction between Leuconostoc and the claimed genera. That was true in 1986, as evidenced by Bergey's Manual of Systematic Bacteriology (1986), and it is true today, as evidenced by Appendix 2 to the second (2006) edition, and by the DSM2 "Bacterial Nomenclature Up-to-Date" (March, 2006).

There may, of course, be a bit of uncertainty "at the fringe". One organism may be confused with another because of improper examination. For example, Bergey's Manual (1986) states that "gas production from glucose will separate the leuconostocs from the streptococci but this properly should be tested only with actively growing strains, otherwise gas production in the former may not be evident (1073-4). It also says that "normal streptococcal media are unsuitable for leuconostocs and if used can result in misidentification owing to growth".

There can also be organisms which in fact have intermediate characteristics so their classification is somewhat arbitrary. (Of course, they should still be distinguishable from the more characteristic species of either genus.)

Nonetheless, the utility of the bacterial taxonomy is generally recognized. Indeed, the PTO uses it for patent classification[1], implying its relevancy.

With regard to issue (2), classification of a claimed organism as a different species was given weight in Novo Industri A/S v. Travenol Laboratories, Inc., 211 USPQ 371 (N.D. Ill), opinion supplemented, 211 USPQ 379 (N.D. Ill. 1981), aff'd 215 USPQ 412 (7th Cir. 1982) (Mucor pusillus versus Mucor miehei). Naturally, higher taxonomic distinctions are given even greater weight, see In re Vaeck, 20 USPQ2d 1438 (Fed. Cir. 1991) (Cyanobacteria versus B. megaterium, B. subtilis and E. coli).

The mere mis-identification or reclassification of a single

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[1] See e.g., 424/93.4-93.48, 435/7.32-7.37, 435/36, 435/221, 435/252.1-253.6, 435/822-910.

strain of Leuconostoc mesenteroides as pediococcus is legally inadequate to suggest use of the claimed genera in place of Leuconostoc.

Absent some suggestion, in the prior art and not obtained by hindsight, the routine worker would not "cross taxonomic lines". The difference in taxonomy would lead the routine worker to expect differences in metabolism which would affect the expectation of success.

Turning to issue (3), the art would have been aware of specific differences in lactic acid metabolism between Leuconostoc and the claimed genera. These would tend to defeat any suggestion, or expectation of success, which even arguably might be created by the classification history.

It is true, of course, that a particular strain doesn't change its metabolic pathways. However, that doesn't mean that there aren't relevant metabolic differences among the lactic acid bacteria, which are formally defined at page 8, lines 23-32.

We will now compare the characteristics of Leuconostoc to those of the claimed genera, and discuss at the same time the alleged cross-classifications.

Leuconostoc are gram-positive cocci, characterized, inter alia, by fermentation of sugar to produce ethanol, D-(-)-lactic acid, and CO_2 (Bergey's Manual of Systematic Bacteriology (1986), p. 1071-1074).

The Examiner contended that Lactobacilli should be grouped with Leuconostocs because the ATCC catalog entry for ATCC 15520 indicated that it was initially identified as Lactobacillus batatas, but is now listed as Leuconostoc lactis (Lactobacillus batatas is not on the list of valid bacterial species names compiled by DSM2 is March 2006, see "Bacterial Nomenclature Up-to-Date, so we presume this the result of a reclassification).

It is not necessary for us to determine whether the genus of Lactobacilli is distinguishable from Leuconostoc (although we

note that in 2006 it was still recognized as a separate genus), because we have amended claim 6 to excise Lactobacillus, without prejudice or disclaimer.

While excising Lactobacillus, we are retaining Bifidobacterium. The Examiner has previously cited ATCC 11863, originally identified as Lactobacillus bifidus and now listed as Bifidobacterium bifidum.

As explained in Bergey's (1986) (p. 1217),

> The rod-shaped bifidobacteria, which until the eighth edition of Bergey's Manual had long been included in the genus Lactobacillus as "Lactobacillus bifidus", may be differentiated from lactobacilli on the basis of their characteristic hexose fermentation pathway which yields lactic acid and acetic acid at a molar ratio of 2:3, but no CO2, instead of lactic acid, acetic acid (or ethanol) and CO2 at a molar ratio of 1:1:1, the pattern of fermentation products typical of obligately heterofermentative lactobacilli.

Bergey's Manual (1986) provides further information on page 1418, namely, that glucose is fermented by bifidobacteria to produce acetic and lactic acid primarily in the molar ratio of 3:2. CO_2 is produced only the degradation of gluconate. Ethanol production is small.

On page 1217, it comments, on the basis of a 16SrRNA dendrogram, "Bifidobacterium, already excluded from the family Lactobacillaceae in Bergey's Manual, eighth edition, have proved completely unrelated to lactobacilli. They belong to the so-called Actinomycetales subbranch of the gram-positive bacteria".

The foregoing quotations plainly defeat any argument that because the Bifidobacteria were once classified as Lactobacilli, and because it can be difficult to distinguish Lactobacilli from Leuconostocs based on morphology, that the routine worker would

substitute Bifidobacteria for the references' Leuconostoc.

Turning to Pediococcus, the Examiner relies on ATCC 8042, initially identified as Leuconostoc mesenteroides, and now listed as Pediococcus acidilactici.

In Pediococci, glucose is fermented to DL or L-(+)-lactate (Bergey's, p. 1075). Again, gas is not formed. This clearly distinguishes Leuconostoc in the critical area of lactic acid metabolism.

Bergey's also comments that pediococci are morphologically distinct from other lactic acid bacteria.

It is not clear to us why ATCC 8042 was first identified as Leuconostoc mesenteroides (Bergey's p. 1074) rather than Pediococcus acidilactici (Bergey's 1079) but we think it inappropriate to infer from a single mistake that the Leuconostoc and Pediococcus genera can be lumped together, given the relevant metabolic and morphologic differences.

The Examiner has not shown any "cross-identification" between (1) Leuconostoc and (2) Lactococcus or Streptococcus.

The Lactococci were at one time known as the "lactic acid streptococci", see Bergey's 1065. The subgroup became a separate genus in 1986, see Schleifer et al., Validation List No. 20, Int. J. Syst. Bacteriol. 36:354-6 (1986).[2] As noted by Bergey's 1043, for streptococci generally, carbohydrates are fermented to produce mainly lactic acid.

Fig. 1 and Table 1 in Starrenburg also evidence significant differences in lactose and citrate metabolism, respectively, between Lactococcus lactis and two Leuconostoc species.

The lactic acid metabolism of Leuconostoc is further compared to that of the claimed genera Lactococcus, Streptococcus, Pediococcus and Bifidobacterium in the enclosed

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[2] This explains the changes in classification of ATCC 7962 and 11007.

Declaration of Eric Johansen.

The distinctions are summarized in the table below:

Leuconostoc	heterofermentative; CO_2 produced
Lactococcus Streptococcus	homofermentative
Pediococcus	homofermentative; no CO_2 production
Bifidobacterium	heterofermentative; CO_2 produced only from gluconate

Conclusion

Thus, there is a clear distinction between Leuconostoc and the claimed genera, which speaks against a finding that it would have been obvious to substitute any of the claimed genera for Leuconostoc. Hence, the rejection should be withdrawn.

Respectfully submitted,

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By: _____
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Enclosure
-Eric Johansen Declaration
-Bergey's Manual of Systematic Bacteriology, part. A, vol. 2 (2d ed. 2005), Appendix 2 (2pp.)
-Bergey's Manual of Systematic Bacteriology (1st ed. 1986), pp. 1071-5, 1079, 1217, 1418
-DSMZ Bacterial Nomenclature Up-to-Date, pp. 13, 37-39, 41, 55, 70-71
-pp. 115-16 of Hoier et al. (1999), cited in the Declaration
-Garvie et al. (1984), cited in the declaration
-Simpson and Taguchi (1995), cited in the declaration

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PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Nilsson, Dan	Examiner:	V. Afremova
Serial #:	10/658,376	Group art unit:	1651
Filed:	20 August 1997	Docket:	
Title:	Metabolic engineered lactic acid bacteria and their use		

DECLARATION BY ERIC JOHANSEN

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

I, Eric Johansen, residing at Gutfeldtsvej 9, Hørsholm, Denmark and employed as Vice President, Molecular Microbiology at Chr Hansen A/S located at 10-12 Bøge Allé, Hørsholm, Denmark, having attained a PhD in microbiology from the Massachusetts Institute of Technology (MIT) and having over 23 years experience as a professional microbiologist hereby declare:

My curriculum vitae is attached.

I, Eric Johansen, have read the office action mailed the 01/05/2006 and the pending claims.

The metabolic pathways of lactic acid bacteria are not the same in different species of lactic acid bacteria. Lactic acid bacteria are generally classified by the fermentation pathway used to ferment glucose and by their cell morphology. However, the lactic acid metabolic pathway, i.e. the way that lactic acid or lactate is produced, differs between lactic acid bacteria species. Two main themes are known. In homofermentative lactic acid bacteria, the primary metabolic end product of sugar fermentation is lactate; other compounds are only produced under exceptional conditions and generally in small amounts compared to the amounts of lactate produced. In heterofermentative lactic acid bacteria, the fermentation products always include compounds in addition to lactate and these are produced in significant amounts; in some cases equimolar amounts compared to lactate levels.

In *Leuconostoc* the lactic acid metabolic pathway is heterofermentative and lactose is metabolized to lactate, ethanol and CO_2 (Table 4.3 on page 115 in Høier, E., T. Janzen, C.M. Henriksen, F. Rattray, E. Brockmann and E. Johansen, (1999),

The production, application and action of lactic cheese starter cultures, in B. Law (ed), The Technology of Cheesemaking, Academic Press, Sheffield, UK, pp 99-131).

In *Lactococcus* and *Streptococcus* the lactic acid metabolic pathway is homofermentative and lactose is metabolised to lactate (Table 4.3 on page 115 in Høier, E., T. Janzen, C.M. Henriksen, F. Rattray, E. Brockmann and E. Johansen, (1999), The production, application and action of lactic cheese starter cultures, in B. Law (ed), The Technology of Cheesemaking, Academic Press, Sheffield, UK, pp 99-131).

In *Pediococcus* the lactic acid metabolic pathway is homofermentative (Simpson WJ and Taguchi H, 1995, The genus *Pediococcus* with notes on the genera *Tetratogenococcus* and *Aerococcus*, pp. 125-172, in Wood BJB and Holzapfel WH (eds.), The genera of Lactic Acid Bacteria, Chapman & Hall, London).

In *Bifidobacterium* the lactic acid metabolic pathway is heterofermentative and is characterised by the production of acetic and lactate in a molar ratio of 3:2. There is no CO_2 produced by this genus except in the case of gluconate degradation. Small amounts of formic acid, ethanol and succinic acid can also be produced by *Bifidobacterium*. Glucose is degraded by this genus characteristically via the fructose-6-phosphate shunt. In this pathway fructose-6-phosphate is cleaved into acetylphosphate and erythrose-4-phosphate by the enzyme fructose-6-phosphoketolase. End products of metabolism are formed by sequential action of transaldolase and transketolase, xylose-5phophate phosphoketolase and enzymes of the Embden-Myerhof pathway action on glyceraldehyde-3-phosphate. Additional acetic and formic acid may be formed via cleavage of pyruvate (Scardovi V (1986), Genus *Bifidobacterium* Orla-Jensen, in Bergey's Manual of Systematic Bacteriology, vol. 2, pp. 1418–1434, Edited by Sneath PHA, Mair NS, Sharpe ME & Holt JG, Baltimore: Williams & Wilkins). This glucose degradation pathway makes *Bifidobacterium* distinct from all the other species of lactic acid bacteria.

Thus the lactic acid metabolic pathway in *Lactococcus*, *Pediococcus*, *Streptococcus* and *Bifidobacterium* are distinct from the lactic acid metabolic pathway in *Leuconostoc*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United State Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name: Eric Johansen Date: month/day/year ¤

June/02/2006

Appendix 2. Taxonomic Outline of the *Archaea* and *Bacteria*

Readers are advised that the taxonomic scheme presented here is a work-in-progress and is based on data available in October 2003. Some rearrangement and emendment is expected to occur as new data become available and subsequent volumes go to press.

Domain *Archaea*[VP]
- Phylum AI. *Crenarchaeota*[VP]
  - Class I. *Thermoprotei*[VP]
    - Order I. *Thermoproteales*[VP (T)]
      - Family I. *Thermoproteaceae*[VP]
        - Genus I. *Thermoproteus*[VP (T)]
        - Genus II. *Caldivirga*[VP]
        - Genus III. *Pyrobaculum*[VP]
        - Genus IV. *Thermocladium*[VP]
        - Genus V. *Vulcanisaeta*[VP]
      - Family II. *Thermofilaceae*[VP]
        - Genus I. *Thermofilum*[VP (T)]
    - Order II. *"Caldisphaerales"*
      - Family I. *"Caldisphaeraceae"*
        - Genus I. *Caldisphaera*[VP (T)]
    - Order III. *Desulfurococcales*[VP]
      - Family I. *Desulfurococcaceae*[VP]
        - Genus I. *Desulfurococcus*[VP (T)]
        - Genus II. *Acidilobus*[VP]
        - Genus III. *Aeropyrum*[VP]
        - Genus IV. *Ignicoccus*[VP]
        - Genus V. *Staphylothermus*[VP]
        - Genus VI. *Stetteria*[VP]
        - Genus VII. *Sulfophobococcus*[VP]
        - Genus VIII. *Thermodiscus*[VP]
        - Genus IX. *Thermosphaera*[VP]
      - Family II. *Pyrodictiaceae*[VP]
        - Genus I. *Pyrodictium*[VP (T)]
        - Genus II. *Hyperthermus*[VP]
        - Genus III. *Pyrolobus*[VP]
    - Order IV. *Sulfolobales*[VP]
      - Family I. *Sulfolobaceae*[VP]
        - Genus I. *Sulfolobus*[AL (T)]
        - Genus II. *Acidianus*[VP]
        - Genus III. *Metallosphaera*[VP]
        - Genus IV. *Stygiolobus*[VP]
        - Genus V. *Sulfurisphaera*[VP]
        - Genus VI. *Sulfurococcus*[VP]
- Phylum AII. *Euryarchaeota*[VP]
  - Class I. *Methanobacteria*[VP]
    - Order I. *Methanobacteriales*[VP (T)]
      - Family I. *Methanobacteriaceae*[AL]
        - Genus I. *Methanobacterium*[AL (T)]
        - Genus II. *Methanobrevibacter*[VP]
        - Genus III. *Methanosphaera*[VP]
        - Genus IV. *Methanothermobacter*[VP]
      - Family II. *Methanothermaceae*[VP]
        - Genus I. *Methanothermus*[VP (T)]
  - Class II. *Methanococci*[VP]
    - Order I. *Methanococcales*[VP (T)]
      - Family I. *Methanococcaceae*[VP]
        - Genus I. *Methanococcus*[AL (T)]
        - Genus II. *Methanothermococcus*[VP]
      - Family II. *Methanocaldococcaceae*[VP]
        - Genus I. *Methanocaldococcus*[VP (T)]
        - Genus II. *Methanotorris*[VP]
  - Class III. *"Methanomicrobia"*
    - Order I. *Methanomicrobiales*[VP (T)]
      - Family I. *Methanomicrobiaceae*[VP]
        - Genus I. *Methanomicrobium*[VP (T)]
        - Genus II. *Methanoculleus*[VP]
        - Genus III. *Methanofollis*[VP]
        - Genus IV. *Methanogenium*[VP]
        - Genus V. *Methanolacinia*[VP]
        - Genus VI. *Methanoplanus*[VP]
      - Family II. *Methanocorpusculaceae*[VP]
        - Genus I. *Methanocorpusculum*[VP (T)]
      - Family III. *Methanospirillaceae*[VP]
        - Genus I. *Methanospirillum*[AL (T)]
      - Genera incertae sedis
        - Genus I. *Methanocalculus*[VP]
    - Order II. *Methanosarcinales*[VP]
      - Family I. *Methanosarcinaceae*[VP]
        - Genus I. *Methanosarcina*[AL (T)]
        - Genus II. *Methanococcoides*[VP]
        - Genus III. *Methanohalobium*[VP]
        - Genus IV. *Methanohalophilus*[VP]
        - Genus V. *Methanolobus*[VP]
        - Genus VI. *Methanimicrococcus*[VP]
        - Genus VII. *Methanosalsum*[VP]
      - Family II. *Methanosaetaceae*[VP]
        - Genus I. *Methanosaeta*[VP (T)]
  - Class IV. *Halobacteria*[VP]
    - Order I. *Halobacteriales*[VP (T)]
      - Family I. *Halobacteriaceae*[AL]
        - Genus I. *Halobacterium*[AL (T)]
        - Genus II. *Haloarcula*[VP]
        - Genus III. *Halobaculum*[VP]
        - Genus IV. *Halobiforma*[VP]
        - Genus V. *Halococcus*[AL]
        - Genus VI. *Haloferax*[VP]
        - Genus VII. *Halogeometricum*[VP]
        - Genus VIII. *Halomicrobium*[VP]
        - Genus IX. *Halorhabdus*[VP]
        - Genus X. *Halorubrum*[VP]
        - Genus XI. *Halosimplex*[VP]
        - Genus XII. *Haloterrigena*[VP]
        - Genus XIII. *Natrialba*[VP]
        - Genus XIV. *Natrinema*[VP]
        - Genus XV. *Natronobacterium*[VP]
        - Genus XVI. *Natrononococcus*[VP]
        - Genus XVII. *Natronomonas*[VP]
        - Genus XVIII. *Natronorubrum*[VP]
  - Class V. *Thermoplasmata*[VP]
    - Order I. *Thermoplasmatales*[VP (T)]
      - Family I. *Thermoplasmataceae*[VP]
        - Genus I. *Thermoplasma*[AL (T)]
      - Family II. *Picrophilaceae*[VP]
        - Genus I. *Picrophilus*[VP (T)]
      - Family III. *"Ferroplasmaceae"*
        - Genus I. *Ferroplasma*[VP]

AL - Approved Lists; VP - validly published; NP - new proposal appearing in Volume Two of *Bergey's Manual of Systematic Bacteriology*, 2nd Edition. PART A

VOL 2, 2005 ISBN-10: 0-387-24143-4

Family I. *Acholeplasmataceae*[AL]
Genus I. *Acholeplasma*[AL]
Order IV. *Anaeroplasmatales*[VP]
Family I. *Anaeroplasmataceae*[VP]
Genus I. *Anaeroplasma*[AL (T)]
Genus II. *Asteroleplasma*[VP]
Order V. *Incertae sedis*
Family I. *"Erysipelotrichaceae"*
Genus I. *Erysipelothrix*[AL]
Genus II. *Bulleidia*[VP]
Genus III. *Holdemania*[VP]
Genus IV. *Solobacterium*[VP]
Class III. *"Bacilli"*
Order I. *Bacillales*[AL]
Family I. *Bacillaceae*[AL]
Genus I. *Bacillus*[AL (T)]
Genus II. *Amphibacillus*[VP]
Genus III. *Anoxybacillus*[VP]
Genus IV. *Exiguobacterium*[VP]
Genus V. *Filobacillus*[VP]
Genus VI. *Geobacillus*[VP]
Genus VII. *Gracilibacillus*[VP]
Genus VIII. *Halobacillus*[VP]
Genus IX. *Jeotgalibacillus*[VP]
Genus X. *Lentibacillus*[VP]
Genus XI. *Marinibacillus*[VP]
Genus XII. *Oceanobacillus*[VP]
Genus XIII. *Paraliobacillus*[VP]
Genus XIV. *Saccharococcus*[VP]
Genus XV. *Salibacillus*[VP]
Genus XVI. *Ureibacillus*[VP]
Genus XVII. *Virgibacillus*[VP]
Family II. *"Alicyclobacillaceae"*
Genus I. *Alicyclobacillus*[VP]
Genus II. *Pasteuria*[AL]
Genus III. *Sulfobacillus*[VP]
Family III. *Caryophanaceae*[AL]
Genus I. *Caryophanon*[AL (T)]
Family IV. *"Listeriaceae"*
Genus I. *Listeria*[AL]
Genus II. *Brochothrix*[AL]
Family V. *"Paenibacillaceae"*
Genus I. *Paenibacillus*[VP]
Genus II. *Ammoniphilus*[VP]
Genus III. *Aneurinibacillus*[VP]
Genus IV. *Brevibacillus*[VP]
Genus V. *Oxalophagus*[VP]
Genus VI. *Thermicanus*[VP]
Genus VII. *Thermobacillus*[VP]
Family VI. *Planococcaceae*[AL]
Genus I. *Planococcus*[AL (T)]
Genus II. *Filibacter*[VP]
Genus III. *Kurthia*[AL]
Genus IV. *Planomicrobium*[VP]
Genus V. *Sporosarcina*[AL]
Family VII. *"Sporolactobacillaceae"*
Genus I. *Sporolactobacillus*[AL]
Genus II. *Marinococcus*[VP]
Family VIII. *"Staphylococcaceae"*
Genus I. *Staphylococcus*[AL]
Genus II. *Gemella*[AL]
Genus III. *Jeotgalicoccus*[VP]
Genus IV. *Macrococcus*[VP]
Genus V. *Salinicoccus*[VP]
Family IX. *"Thermoactinomycetaceae"*
Genus I. *Thermoactinomyces*[AL]
Family X. *"Turicibacteraceae"*
Genus I. *Turicibacter*[VP (T)]
Order II. *"Lactobacillales"*
Family I. *Lactobacillaceae*[AL]
Genus I. *Lactobacillus*[AL (T)]
Genus II. *Paralactobacillus*[VP]
Genus III. *Pediococcus*[AL]
Family II. *"Aerococcaceae"*
Genus I. *Aerococcus*[AL]
Genus II. *Abiotrophia*[VP]
Genus III. *Dolosicoccus*[VP]
Genus IV. *Eremococcus*[VP]
Genus V. *Facklamia*[VP]
Genus VI. *Globicatella*[VP]
Genus VII. *Ignavigranum*[VP]
Family III. *"Carnobacteriaceae"*
Genus I. *Carnobacterium*[VP]
Genus II. *Agitococcus*[VP]
Genus III. *Alkalibacterium*[VP]
Genus IV. *Allofustis*[VP]
Genus V. *Alloiococcus*[VP]
Genus VI. *Desemzia*[VP]
Genus VII. *Dolosigranulum*[VP]
Genus VIII. *Granulicatella*[VP]
Genus IX. *Isobaculum*[VP]
Genus X. *Lactosphaera*[VP]
Genus XI. *Marinilactibacillus*[VP]
Genus XII. *Trichococcus*[VP]
Family IV. *"Enterococcaceae"*
Genus I. *Enterococcus*[VP]
Genus II. *Atopobacter*[VP]
Genus III. *Melissococcus*[VP]
Genus IV. *Tetragenococcus*[VP]
Genus V. *Vagococcus*[VP]
Family V. *"Leuconostocaceae"*
Genus I. *Leuconostoc*[AL]
Genus II. *Oenococcus*[VP]
Genus III. *Weissella*[VP]
Family VI. *Streptococcaceae*[AL]
Genus I. *Streptococcus*[AL (T)]
Genus II. *Lactococcus*[VP]
Family VII. *Incertae sedis*
Genus I. *Acetoanaerobium*[VP]
Genus II. *Oscillospira*[AL]
Genus III. *Syntrophococcus*[VP]
Phylum BXIV. *"Actinobacteria"*[NP]
Class I. *Actinobacteria*[VP]
Subclass I. *Acidimicrobidae*[VP]
Order I. *Acidimicrobiales*[VP]
Suborder I. *"Acidimicrobineae"*
Family I. *Acidimicrobiaceae*[VP]
Genus I. *Acidimicrobium*[VP (T)]
Subclass II. *Rubrobacteridae*[VP]
Order I. *Rubrobacterales*[VP]
Suborder I. *"Rubrobacterineae"*
Family I. *Rubrobacteraceae*[VP]
Genus I. *Rubrobacter*[VP (T)]
Genus II. *Conexibacter*[VP]

with clear centers and granular peripheries. All strains require some CO_2, but some are inhibited by concentrations over 5% (v/v). Glucose and fructose are usually the only sugars to support growth; some strains dissimilate sucrose, melezitose and salicin. Major amounts of lactic acid are produced; small amounts of acetic, isobutyric and succinic acids also produced. Final pH, 5.3. Optimum growth at 35°C; some growth between 20° and 45°C. Optimum pH, 6.5–6.6. Requires a Na:K ratio of 1. Requires free cysteine or cystine in addition to peptones or most yeast extracts; little or no growth on most ordinary media. Reacts with Lancefield Group D antiserum.

The cell wall peptidoglycan type is Lys-Ala. The major long chain fatty acids are hexadecanoic and lactobacillic acids.

Causative agent of European foulbrood of the honeybee. Isolated from larvae of *A. millifera* and *A. cerana* with European foulbrood.

The mol% G + C of the DNA is 29–30% (T_m).

Type strain: NCDO 2443

Further taxonomic comments. The descriptions given above are based on those published by Bailey and Collins (1982a, b). Comparative studies of *M. pluton* with other Gram-positive cocci, using modern numerical, chemical or genetic taxonomic techniques are not available.

Genus Leuconostoc *van Tieghem 1878, 198[AL] emend mut. char. Hucker and Pederson 1930, 66[AL]*

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Leu.co.nos'toc. Gr. Adj. *leucus* clear, light; M.L. neut. n. *Nostoc* algal generic name; M.L. neut. n. *Leuconostoc* colorless nostoc.

Cells may be spherical but often lenticular particularly when growing on agar, cells usually occur in pairs and chains. Gram-positive, nonmotile, spores not formed. Facultative anaerobes.

Colonies are small usually less than 1.00 mm in diameter, smooth, round, grayish white. In stab cultures growth occurs along the stab with little surface growth. Broth cultures often have uniform turbidity but strains forming long chains tend to sediment. Optimum temperature 20–30°C and growth occurs between 5°C and 30°C. Chemoorganotrophs, requiring a rich medium often having complex growth factors and amino acid requirements. All species require nicotinic acid + thiamine + biotin and either pantothenic acid or a pantothenic acid derivative. No strains require cobalamin, or *p*-aminobenzoic acid.

Growth is dependent on the presence of a fermentable carbohydrate and glucose is fermented by a combination of the hexose-monophosphate and phosphoketolase pathways. However, the pathway of glucose fermentation in *Leuconostoc oenos* has not been fully confirmed. Fructose 1,6-diphosphate aldolase is absent, and an active glucose-6-phosphate dehydrogenase is present. CO_2 and D-ribulose-5-P are formed from glucose. Xylulose 5-P phosphoketolase is present and the resulting end products are ethanol and D-(−)-lactic acid. Some strains have an oxidative mechanism and acetic acid is formed in place of ethanol. Polysaccharides and alcohols (except mannitol) are usually not fermented. Malate can be utilized and converted to L-(+)-lactate.

Catalase-negative. Cytochromes are absent. Arginine is not hydrolyzed and milk is usually not acidified and curdled. Nonproteolytic. Indole is not formed. Nitrates not reduced. Nonhemolytic. Nonpathogenic to plants and animals (including humans). Properties separating the species are given in Table 12.26 and further information is given in Table 12.27.

The amino acid composition of the cross-linking peptide of the cell wall peptidoglycan is of the alanine, serine, lysine type (Table 12.28). The mol% G + C in the DNA is 38–44 (T_m and Bd) (Table 12.29). The type species is *Leuconostoc mesenteroides* (Tsenkovskii) van Tieghem 1878, 191.

Further Descriptive Information

Growth conditions may affect cell morphology, and not all strains will be influenced in the same way. Cultured in milk (or supplemented milk), most strains form coccoid cells in chains. Chain length varies with the strain. Cultured in broth, cells are elongated and can be mistaken for rods, appearing morphologically closer to the lactobacilli than to the streptococci. Cultured on agar, spherical cells are seldom formed.

The cell wall of dextran-forming strains contains dextran-sucrase and the cell wall structure is affected by growth in sucrose broth, to which strains differ in their response (Brooker, 1976, 1977). Although capsular material is apparent in some strains, a true bacterial capsule is not formed.

The composition of the cross-linked peptide in the cell wall peptidoglycan is given in Table 12.28.

Growth is never rapid, the active strains of *L. mesenteroides* subsp. *mesenteroides* have the shortest generation time and good growth can be obtained in 24 h incubation at 30°C. On the other hand, *L. mesen-*

Table 12.26.
Diagnostic characteristics of the species of the genus Leuconostoc[a]

Characteristics	1. *L. mesenteroides*, subsp.[b]			2. *L. paramesenteroides*	3. *L. lactis*	4. *L. oenos*
	1a. *mesenteroides*	1b. *dextranicum*	1c. *cremoris*			
Acid from						
Arabinose	+	−	−	d	−	d
Cellulose	d	d	−	(d)	−	d
Fructose	+	+	−	+	+	+
Sucrose	+	+	−	+	+	−
Trehalose	+	+	−	+	−	+
Hydrolysis of esculin	d	d	−	d	−	+
Dextran formation	+	+	−	−	−	−
Growth at pH 4.8	−	−	−	d	−	+
Requirement for TJF	−	−	−	−	−	d
Growth in 10% ethanol	−	−	−	−	−	+
NAD-dependent G-6-PDH present	+	+	+	+	+	−

[a] Symbols: see Table 12.2; also (d) delayed reaction; and TJF, glucopantothenate (tomato juice factor).

Table 12.27.
Differential characteristics of the species of the genus **Leuconostoc**[a,b]

Characteristics	1. *L. mesenteroides*, subsp.			2. *L. paramesenteroides*	3: *L. lactis*	4: *L. oenos*
	1a. *mesenteroides*	1b. *dextranicum*	1c. *cremoris*			
Acid from						
Amygdalin	d	d	−	(d)	−	ND
Arabinose	+	−	−	d	−	d
Arbutin	d	−	−	−	−	ND
Cellobiose	d	d	−	(d)	−	d
Fructose	+	+	−	+	+	+
Galactose	+	d	d	+	+	d
Glucose	+	+	+	+	+	+
Lactose	(d)	+	+	(d)	+	−
Maltose	+	+	d	+	+	−
Mannitol	d	d	−	(d)	−	−
Mannose	+	d	−	+	d	d
Melibiose	d	d	−	+	d	d
Raffinose	d	d	−	d	d	−
Ribose	+	ND	ND	ND	ND	ND
Salicin	d	d	−	−	d	d
Sucrose	+	+	−	+	+	−
Trehalose	+	+	−	+	−	+
Xylose	d	d	−	d	−	d
Hydrolysis of esculin	d	d	−	d	−	+
Required for growth						
Uracil	−	−	+	−	−	−
Guanine + adenine + xanthine + uracil	−	d	+	d	−	+
Riboflavin	d	d	+	+	+	+
Pyridoxal	d	d	+	+	−	+
Folic acid	d	d	+	+	−	+
Tomato juice factor	−	−	−	−	−	d
Destruction of tomato juice factor	−	−	−	−	−	d
Dextran formation	+	+	−	−	−	−
Dissimilate citrate (carbohydrate present)	d	d	+	d	d	d
Dissimilate malate						
No carbohydrate present	d	−	−	d	−	ND
Carbohydrate	d	−	−	d	−	+
Yeast glucose litmus milk	+	+	+	+	+	d
Acid clot	d	d	d	d	d	−
Reduction	d	d	−	d	(d)	d
Gas	d	d	−	−	−	−
Growth in						
3.0% NaCl	+	d	−	d	d	ND
6.5% NaCl	d	−	−	d	−	ND
Growth at pH						
4.8 (initial)	−	−	−	d	−	+
6.5 (initial)	+	+	+	+	+	d
Growth at 37°C	d	+	−	d	+	d
Final pH in glucose broth	4.5	4.5	5.0	4.4	4.7	ND

[a] Symbols: see Table 12.2; (d), delayed reaction; TJF, glucopantothenate (tomato juice factor); ND, not determined.
[b] DNA/DNA hybridization shows that strains previously classified as *L. dextranicum* belong to the same genotype as *L. mesenteroides* NCDO 523. This may explain the failure to find satisfactory properties for separating these groups.

teroides subsp. *cremoris* may require 48 h incubation and prefers 22°C to 30°C. The slower growing strains prefer reducing conditions and 0.05% cysteine HCl added to broth media encourages growth. *L. oenos* has many differences from other species and grows best in acid media (initial pH 4.2–4.8) containing tomato juice. Growth is slow and 5–7 days incubation at 22°C may be needed. Other species of leuconostoc will not grow in the acid media preferred by *L. oenos*.

Milk is a poor medium for leuconostocs, although most strains will grow in milk supplemented with yeast extract and glucose. *L. mesenteroides* subsp. *mesenteroides* usually acidifies and clots milk media with gas formation, while other species are less active and *L. paramesenteroides* and other species with a high requirement for amino acids fail to clot milk. Nutritional requirements vary (Garvie, 1967b) Table 12.30). *L. oenos* will grow with a very high level of pantothenic acid but prefers a gluco-derivative of the vitamin, probably 4′o-(β-glucopyranosyl)D-pantothenic acid (Amachi et al. 1970). The degree of depend-

Table 12.28.
Amino acid sequence of the interpeptide bridge of cell wall peptidoglycan of the species of the genus **Leuconostoc**[a]

Species	Peptidoglycan
1a. *L. mesenteroides* subsp. *mesenteroides*	L-Lys-L-Ser-L-Ala_2; L-Lys-L-Ala_2
1b. *L. mesenteroides* subsp. *dextranicum*	L-Lys-L-Ser-L-Ala_2
1c. *L. mesenteroides* subsp. *cremoris*	L-Lys-L-Ser-L-Ala_2
2. *L. paramesenteroides*	L-Lys-L-Ser-L-Ala_2; L-Lys-L-Ala_2
3. *L. lactis*	L-Lys-L-Ser-L-Ala_2; L-Lys-L-Ala_2
4. *L. oenos*	L-Lys-L-Ala-L-Ser; L-Lys-L-Ser-L-Ser

[a] Adapted from W. H. Holzapfel, Inaugural dissertation der Technischen Hochschule, München, 1969.

Table 12.29.
Mol% G + C of the DNA of the **Leuconostoc** *species*[a]

Species	T_m and buoyant density
1. *L. mesenteroides*	
a. Subsp. *mesenteroides*	37–39, with some strains 40–41
b. Subsp. *dextranicum*	37–40
c. Subsp. *cremoris*	38–40
2. *L. lactis*	43–45
3. *L. paramesenteroides*	37–38
4. *L. oenos*	37–39

[a] Compiled from Garvie et al. (1974) and Hontebeyrie and Gasser (1977).

ence on this growth factor varies with different strains, but most strains will destroy it when present in media. Other species of *Leuconostoc* do not attack glucopantothenic acid (Garvie and Mabbitt, 1967).

L. mesenteroides subsp. *mesenteroides* requires only glutamic acid and valine while other subspecies and species require a variety of amino acids, the requirements varying with the strain (Garvie, 1967b).

Leuconostocs are dependent on the presence of a fermentable carbohydrate, and fermentation ability varies in different species (Table 12.27). Glucose is used by all species but fructose is prefered by all except *L. mesenteroides* subsp. *cremoris*. Glucose is phosphorylated and all species have an active glucose-6-phosphate dehydrogenase (G-6-PDH). In species 1–3, NAD or NADP will serve as coenzyme with a preference for the former but in *L. oenos* the G-6-PDH is active only with NADP (Garvie, 1975). Gluconate is decarboxylated and pentose converted to D-(−)-lactate and ethanol by the phosphoketolase pathway. Acetate as well as ethanol may be formed by some strains. The D-(−)-lactate dehydrogenase (LDH) of *L. oenos* migrates slowly on electrophoresis and there is evidence of differences between strains. The LDH of other species has a fast migration which is the same for them all (Garvie, 1969). Immunological studies have separated the LDHs and G-6-PDHs of different species. More than one type of these enzymes has been found in different strains of *L. mesenteroides* subsp. *mesenteroides*, indicating that it is a heterologous species (Hontebeyrie and Gasser, 1975).

Early work indicated that most leuconostocs dissimilate citrate (Hucker and Pederson, 1930). This property appears to be lost in strains kept in the laboratory but is important in strains which are components of cheese and butter starters (species 1c and 3). *L. oenos* can also dissimilate citrate. Malate is attacked by *L. oenos* and also by *L. mesenteroides* subsp. *mesenteroides*. Information on other species is lacking. Malate is converted to L-(+)-lactate, and an LDH is not involved (Alizade and Simon, 1973; Radler, 1975). It is important to exclude malate from media used for cultures when the type of lactic acid produced from glucose is to be determined. Acetate and tartrate are not utilized.

The mol% G + C in the DNA of *L. lactis* is 43–45 while that of other species is 37–40. There are indications from the values obtained that *L. mesenteroides* subsp. *mesenteroides* is a heterologous subspecies (Garvie et al. 1974; Hontebeyrie and Gasser, 1977). This situation is confirmed by DNA/DNA hybridization studies (Garvie, 1976; Hontebeyrie and Gasser, 1977). In addition high hybridization occurs between the DNA of the three species, *L. mesenteroides*, *L. dextranicum* and *L. cremoris*, showing that they belong to a single genospecies, as put forward by Garvie (1983). The other species are clearly identified (Table 12.31). RNA/DNA hybridization separates *L. oenos* from the other leuconostocs which all belong to a single RNA group (Garvie, 1981).

Phage attack on *L. oenos* may occur in wine making, and bacteriophages have been described (Sozzi et al. 1982). Bacteriophage for *L. mesenteroides* is reported (Sozzi et al. 1978), but very little is known about bacteriophages attacking leuconostocs.

Sensitivity to antibiotics and drugs is unknown as no species are pathogenic.

Leuconostocs are found on plants and to a lesser extent in milk and milk products. *L. mesenteroides* subsp. *cremoris* and *L. lactis* may be components of cheese and butter starters. Dextran-forming species occur on sugar cane and sugar beet where they may cause widespread spoilage. *L. oenos* is known only in wine and related habitats; no other leuconostoc has been isolated from these sources.

Enrichment and Isolation Procedures

Leuconostocs on plants can be isolated on media containing thallous acetate and crystal violet (Cavett et al. 1965). Enrichment in broth may be necessary before plating on agar. Citrate-utilizing strains in dairy starters can be isolated on whey agar (Galesloot et al. 1961) and *L. oenos* can be isolated on tomato juice agar with initial pH below 4.5 with cyclohexamide to inhibit yeasts (Kunkee, 1967). Growth of *L. oenos* on agar may be poor and growth in broth often slow.

Maintenance Procedures

All species can be preserved by lyophilization in horse serum + 7.5% glucose, but cells of an actively growing culture in late logarithmic or early stationary phase should be used. Care is needed with *L. mesenteroides* subsp. *cremoris* and *L. oenos*. It is important to wait for high turbidity in a culture before lyophilization. Once dried, cultures can be kept under vacuum at 10°C. Cultures should be revived in media giving optimum growth conditions.

Nonacidophilic species can be kept for 3–4 months in litmus milk + 0.3% yeast + 1% glucose and 1% calcium carbonate. Preliminary incubation for 18–24 or 48 h (depending on the strain) at 30°C is necessary before storage. *L. oenos* can be kept in tomato juice agar stabs.

Taxonomic Comments

Leuconostocs occur in the same habitats as lactobacilli and lactic streptococci. Gas production from glucose will separate the leuconos-

Table 12.30.
Growth factor requirements of the **Leuconostoc** *species*[a]

Characteristics	1. *L. mesenteroides*, subsp.			2. *L. paramesenteroides*	3. *L. lactis*	4. *L. oenos*
	1a. *mesenteroides*	1b. *dextranicum*	1c. *cremoris*			
Uracil	−	−	+	−	−	−
Guanine + adenine + xanthine + uracil	−	−	+	d	−	+
Riboflavin	d	d	+	+	+	+
Pyridoxal	d	d	+	d	−	−
Folic acid	d	d	+	+	−	+
Tween 80	−	d	−	d	−	d

[a] Symbols: see Table 12.2.

Table 12.31.
DNA/DNA homology between the species of **Leuconostoc**[a,b]

Species		Labeled DNA from			
		L. mesenteroides		*L. lactis*	*L. paramesenteroides*
L. mesenteroides	A	85–100	35–50	20–50	8–22
	B	46–78	90–100	20–50	8–22
L. dextranicum		85–100	38–50	19–30	7–19
L. cremoris		78–100	46	24–35	5
L. lactis		32–47	40–60	70–100	9–46
L. paramesenteroides		11–16	17–21	6–14	60–100
L. oenos		11–15	10	2–11	5

[a] There are indications that the *L. mesenteroides*/*L. dextranicum* group contains more than two genotypes.
[b] Compiled from Garvie (1976) and Hontebeyrie and Gasser (1977).

tocs from streptococci but this property should be tested only with actively growing strains, otherwise gas production in the former may not be evident. Normal streptococcal media are unsuitable for leuconostocs and if used can result in misidentification owing to poor growth. Type of lactic acid produced also separates the D-(−)-forming leuconostocs from L-(+)-forming streptococci.

Separation of leuconostocs from gas-forming lactobacilli is not easy (Sharpe et al. 1972). Morphology can overlap. *Lactobacillus viridescens* does not hydrolyze arginine, forms predominantly D-(−)-lactate but some L-(+)-is usually found, and has a mol% G + C in its DNA between that of *L. mesenteroides* and *L. lactis*. Generally cells of *Lactobacillus viridescens* are more elongated than those of any leuconostoc. *Lactobacillus confusus* usually hydrolyzes arginine and has a mol% G + C in its DNA similar to that of *L. lactis*, and forms DL-lactate.

Early classification relied heavily on morphology and the leuconostocs, being more coccoid than rod-like were placed with the streptococci, while the heterofermentative species with cells more rod-like than coccoid (heterofermentative lactobacilli) were placed with the homofermentative lactobacilli. The significance of the physiological similarities between the leuconostocs and heterofermentative lactobacilli, in particular *Lactobacillus confusus* and *Lactobacillus viridescens*, require reassessing. These latter organisms have similar LDHs and cell wall peptides to leuconostocs but belong to different DNA and RNA homology groups. However, the nonacidophilic leuconostocs *mesenteroides*, *paramesenteroides* and *lactis* appear to have more in common with *Lactobacillus confusus* and *Lactobacillus viridescens* than with *L. oenos*.

The nomenclature of the genus, discussed in detail in the last edition has been modified because of the results of enzyme studies and DNA/DNA homology. These studies have shown that *L. mesenteroides* contains three subspecies viz. *mesenteroides*, *dextranicum* and *cremoris*. The change in status of *L. dextranicum* and *L. cremoris* is discussed elsewhere (Garvie, 1984).

Further Reading

Garvie, E.I. 1984. Separation of species of the genus *Leuconostoc* and the differentiation of the leuconostocs from other lactic acid bacteria. Methods Microbiol. *16*: 147–178.

List of species of the genus **Leuconostoc**

1. Leuconostoc mesenteroides (Tsenkovskii) van Tieghem 1879, 198.[AL] (*Ascococcus mesenteroides* Tsenkovskii 1878, 159.)

me.sen.ter.oi'des. Gr. n. *mesenterium* the mesentery; Gr. n. *oides* form, shape; M.L. adj. *mesenteroides* mesentery-like.

Morphology as in general description.

Some strains produce a heme-requiring catalase (Whittenbury, 1964).

In glucose broth cells do not survive heating to 55°C for 30 min but in slimy sugar solutions they may withstand heating to 80–85°C.

Temperature range 10–37°C, optimum 20–30°C.

The mol% G + C of the DNA: see Table 12.29.

Type strain: ATCC 8293 (NCDO 523).

1a. *Leuconostoc mesenteroides* subsp. *mesenteroides* (Tsenkovskii) van Tieghem 1879 198.[AL] A characteristic slime of dextran is formed from sucrose, the production being favored by growing at 20–25°C. Different colonial types are formed on sucrose agar depending on the characteristic chemical structure of the dominant type of dextran formed. These differences have not proved to be of taxonomic value.

1b. *Leuconostoc mesenteroides* subsp. *dextranicum* (Beijerinck) Garvie 1983, 118.[VP] (*Leuconostoc dextranicum* (Beijerinck) Hucker and Pederson 1930, 67; *Lactococcus dextranicus* Beijerinck 1912, 27.)

dex.tra'ni.cum. M.L. n. *dextranum* dextran; M.L. neut. adj. *dextranicum* relating to dextran.

Morphology as in general description.

Dextran is formed but less actively than with *L. mesenteroides* subsp. *mesenteroides*.

This subspecies ferments fewer substrates than subspecies *mesenteroides* and requires a few more amino acids and vitamins for growth.

Optimum growth temperatures and range are the same as for 1a.

Differentiation between *L. mesenteroides* subsp. *mesenteroides* and subsp. *dextranicum* has always been blurred and unsatisfactory. This is probably because they are a single genospecies.

Type strain: NCDO 529 (ATCC 19255).

1c. *Leuconostoc mesenteroides* subsp. *cremoris* (Knudsen and Sorensen) Garvie 1983, 118.[VP] (*Leuconostoc cremoris* (Knudsen and Sorensen) Garvie 1960, 288; *Betacoccus cremoris* Knudsen and Sörensen 1929, 81.)

cre.mor'is. L. n. *cremor* cream; L. gen. no. *cremoris* of cream.

Morphology as general description, but cultures often form long chains with resultant flocculent growth in broth.

Citrate is normally dissimilated and under certain conditions acetoin and diacetyl are formed (Speckman and Collins, 1968). These end products are not always detected because the pyruvate formed from citrate is probably used for the regeneration of NAD, and D-(−)-lactate results.

Most strains do not attack sucrose but mutant colonies in soft agar cultures have been reported (Whittenbury, 1966).

This subspecies is the least active and requires a large number of vitamins and amino acids. It prefers reducing conditions and a temperature of 18–25°C for growth.

It appears to be an adaption of *L. mesenteroides* subsp. *mesenteroides* to the dairy environment. All known strains have come from milk, dairy starter or related habitats. Truly wild sources are unknown.

Difficulties in separating some strains of *L. mesenteroides* subsp. *cremoris* from subsp. *dextranicum* are probably due to the fact that they belong to the same genospecies.

Type strain: NCDO 543 (ATCC 19254).

2. **Leuconostoc paramesenteroides** Garvie 1967b, 446.[AL]

pa.ra.me.sen.ter.oi'des. Gr. prep. *para* resembling; M.L. *mesenteroides* a specific epithet; M.L. adj. *paramesenteroides* resembling *L. mesenteroides*.

Morphology as general description.

Dextran is not formed from sucrose and amino acid requirements are complex and variable.

Many strains grow well at 30°C but some prefer reducing conditions and a temperature of 18–24°C (Garvie, 1967). Pseudocatalase may be present if organisms are grown in a medium with a low glucose content (Whittenbury, 1964).

Tolerance of NaCl is higher than for other species, particularly those strains isolated from foods containing high levels of salt.

More tolerant of acid pH than species 1 or 3 and may grow in media with an initial pH below 5.0.

At one time strains were considered to be nondextran-forming variants of *L. mesenteroides* but genetic studies have shown this to be incorrect. However, it would be difficult to distinguish *L. paramesenteroides* from nondextran-forming strains of *L. mesenteroides* by phenotypic tests.

Type strain: NCDO 803.

3. **Leuconostoc lactis** Garvie 1960, 290.[AL]

lac'tis. L. n. *lac* milk; L. gen. n. *lactis* of milk.

Morphology as general description.

The amino acid requirements are complex. However, lactose is fermented more readily than by other species and strains may acidify and even clot unsupplemented milk.

Citrate may be dissimilated and acetoin and diacetyl formed (Cogan et al., 1981).

Heat resistance is higher than in other species and cells may survive 60°C for 30 min.

The species may not be widely distributed as recorded isolations are few and are mostly from dairy sources.

Type strain: NCDO 533 (ATCC 19256).

4. **Leuconostoc oenos** Garvie 1967a, 431.[AL]

oe.nos. Gr. n. *oinos* wine; Gr. gen. n. *oenos* of wine.

Morphologically resembles the other species but is different in many other respects.

Growth is slow and variations in properties between strains may be due in part to unsuitable growth conditions. Division of wine leuconostocs into separate species has been proposed but a variety of strains from these proposed species have been found to belong to a single genospecies (Garvie and Farrow, 1981). Differences in LDHs in different strains have been found and the species may not be homologous.

Isolated only from wine and related habitats.

Type strain: NCDO 1674 (ATCC 23279).

Genus **Pediococcus** *Claussen 1903, 68*[AL]

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Pe.di.o.coc'cus. Gr. n. *pedium* a plane surface; Gr. n. *coccus* a berry; M.L. masc. n. *Pediococcus*, coccus growing in one plane.

Cells spherical, never elongated, division occurs alternately in two planes at right angles (Günther, 1959) **to form tetrads,** however, these may not always be present and only pairs of cells occur. **Single cells are rare and chains of cells are not formed. Gram-positive, nonmotile. Spores not formed. Facultative anaerobes, but** tolerance to oxygen varies in different species.

Colonies vary in size from 1.0–2.5 mm in diameter, smooth, round, grayish white. In stab culture, growth is along the stab with little [illegible] cultures usually have uniform turbidity. All

Growth is dependent on the presence of a fermentable carbohydrate and glucose is fermented, probably by the Embden-Meyerhof pathway, to DL or L-(+) lactate. Gas is not formed. Under certain growth conditions end products in addition to lactate can be formed.

Catalase-negative. Cytochromes are absent. Milk usually not acidified or curdled. Nonproteolytic. Indole not formed. Nitrates not reduced. Sodium hippurate not hydrolyzed. Nonpathogenic to plants and animals.

[illegible] G + C in the DNA is in the range 34–42. (T_m).

3. **Pediococcus inopinatus** Back 1978a, 245.[VP]

in.o.pin.a'tus. L. adj. *inopinatus* unexpected.

Morphology as general description.

On agar, growth is slow and colonies may take 3–5 days to develop. The final pH in MRS broth is about 4.0.

There is a close similarity between *P. inopinatus* and *P. parvulus* which both occur in the same habitat. DNA/DNA hybridization showed some relationship between the two species and also to *P. damnosus* (Back and Stackebrandt 1978).

The mol% G + C of the DNA is 39–40 (T_m) which is close to that of the other pediococcus species (W. Back, unpublished).

Further separation of *P. parvulus* and *P. inopinatus* was obtained by the electrophoresis of the L-(+)- and D-(−)-LDHs (Back, 1978a).

Habitat: fermenting vegetables and beverages (beer and wine).

Type strain: DSM 20285.

4. **Pediococcus dextrinicus** (Coster and White) Back 1978b, 523.[AL] (*Pediococcus cerevisiae* subsp. *dextrinicus* Coster and White 1964, 29.)

dex.trin'i.cus. M.L. n. *dextrinosum*—dextrin. M.L. neut. adj. *dextrinicus*—relating to dextrin.

Morphology as general description. Less anaerobic than previously described species. Colonies will develop on agar aerobically but growth is improved in an atmosphere of H_2 + 10% CO_2.

In MRS broth the final pH is about 4.4

Optimum pH for growth 6.5. Optimum temperature 30–35°C.

Growth requirements have not been studied. Growth occurs in weakly hopped beer.

Habitat: fermenting vegetables and beer.

The mol% G + C of the DNA is 40–41 (T_m) (Back, 1978b).

Type strain: DSM 20335 (NCDO 1561; ATCC 33087).

5. **Pediococcus pentosaceus** Mees 1934, 96.[AL]

pen.to.sa'ce.us. M.L. neut. n. *pentosum*—a pentose sugar. M.L. adj. *pentosaceus*—relating to a pentose.

Morphology and colonial appearance as general description.

Anaerobic incubation is not necessary and colonies should be visible on agar after incubating aerobically for 24 h at 30°C.

Litmus milk reactions are variable and may be related to growth requirements. The requirement for folinic acid varies between strains.

A limited study of the aldolases found in pediococci have shown that *P. pentosaceus* and *P. acidilactici* are closely related species which are separate from *P. parvulus*. There is some evidence that not all strains of *P. pentosaceus* have the same aldolase (London and Chance, 1976).

In broth growth can be very rapid and the final pH in MRS broth is usually below 4.0. Optimum pH 6.0–6.5. Optimum temperature 28–32°C. Low heat resistance, cells being destroyed at 65°C in 8 min.

The rapid growth, low final pH and absence of cytochromes distinguish *P. pentosaceus* from micrococci. *P. pentosaceus* could be confused with micrococci as it can form small colonies on sugar-free agar and can grow at pH 9.0. It may also be weakly catalase-positive when grown in a medium with low glucose content (Whittenbury 1964).

The mol% G + C of the DNA is 35–39 (T_m) (various authors).

Type strain: NCDO 990 (ATCC 33161; DSM 20336).

6. **Pediococcus acidilactici** Lindner 1887, 440.[AL]

a.ci.di.lac.ti'ci. M.L. n. *acidium lacticum* lactic acid. M.L. gen. n. *acidilactici* of lactic acid.

Morphological, cultural and physiological properties do not readily separate *P. acidilactici* from *P. pentosaceus*.

Optimum temperature of growth 40°C.

Heat tolerant, destroyed at 70°C in 10 min while some strains may be even more heat tolerant particularly when freshly isolated.

The mol% G + C of the DNA is 38–44 (T_m) (various authors). DNA/DNA hybridization shows *P. acidilactici* as a distinct species (Back and Stackebrandt 1978; Dellaglio et al., 1981), while studies of aldolases suggest that *P. pentosaceus* and *P. acidilactici* are closely related.

Type strain: NCDO 1859. The reference strain used by Dellaglio et al., 1981) was ATCC 25742. The work on DNA/DNA hybridization suggests that NCDO 1859, the type strain of *P. acidilactici* is a strain of *P. pentosaceus*. The Judicial Commission should be asked to replace NCDO 1859 with a more suitable strain.

In early work *P. pentosaceus* and *P. acidilactici* were not separated and the properties given may be a combined study of both species. DNA and enzyme studies clearly separate the two species, but when these characteristics cannot be determined difficulties could still arise.

7. **Pediococcus halophilus** Mees 1934, 96.[AL]

hal.o.phi'lus. Gr. n. *halos*, salt. Gr. Adj. *philus*, loving. M.L. adj. *halophilus*, salt loving.

Morphology as general description.

Growth on agar is slow and colonies develop aerobically.

Growth in broth is also slow and 4–5 days incubation may be required. The final pH is about 5.0 and turbidity is less than more acid-tolerant species. Media suitable for the acid-tolerant species do not support good growth of *P. halophilus*, which has an optimum pH for growth between 7.0 and 8.0 and optimum temperature 30–35°C.

Growth will take place in 18% NaCl, and 20–26% may be tolerated (Sakaguchi 1958).

The ratio of D-(−) : L-(+) lactate formed by cultures growing on glucose is about 3:97.

Growth takes place in hopped wort at pH above 5.5 (Sakaguchi, 1958).

The mol% G + C of the DNA is 34–36% (T_m) (various authors).

Type strain: NCDO 1635 (ATCC 33315; DSM 20339).

Comment: In a comparative study of the salt-tolerant pediococci and some aerococci, Deibel and Niven (1960) found that the tetracocci from brine were the same as *P. halophilus* but considered the strains to be *Aerococcus homari* (*Pediococcus homari*, *Aerococcus viridans*). Clearly the relationship between these species requries clarification, as does their relationship to *P. urinaeequi* (see comment after *P. urinaeequi*).

8. **Pediococcus urinaeequi** (ex Mees) nom. rev.

u.ri'nae.e.qui. L. fem. n. *urina*—urine. L. mas. n. *equs*—horse. M.L. gen. n. *urinae-equi*, horse urine.

Morphology as general description.

Growth is generally improved if the initial pH of the medium is alkaline. Optimum pH is between 8.5 and 9.0 (Nakagawa and Kitahara, 1959) although growth will take place in media with an initial pH of 6.5–7.0. The final pH is about 5.0 (Gunther and White, 1961). Optimum temperature 25–30°C. Growth can occur in media which do not contain added carbohydrate (Sakaguchi and Mori, 1969).

L-(+)-lactate is formed from glucose. The LDHs have not been studied. It is not known whether a trace of D-(−)-lactate is formed, as with *P. halophilus*.

The mol% G + C of the DNA is 39.5% (T_m) (Sakaguchi and Mori, 1969; Dellaglio et al. 1974).

The species does not appear to be widely distributed and reported isolations are few.

Type strain: NCDO 1636 (ATCC 29723; DSM 20341).

Comment: The taxonomic position of *P. urinaeequi* requires clarification. There are similarities with *P. halophilus* but the composition of the cell wall murein and salt tolerance are different. Gunther and White (1961) placed *P. urinaeequi* as a variant of their *P. cerevisiae* but Whittenbury (1965) grouped it with *Aerococcus viridans*. It is now known that these species have the same cross-linkage in their cell wall peptide. There is no DNA/DNA homology between *P. urinaeequi* and either *P. halophilus* or *A. viridans* (Dellaglio et al. 1981). However, two strains identified as *Pediococcus homari* (*A. viridans*) had high DNA homology with *P. urinaeequi*.

More work is necessary to clarify the taxonomy of the alkaline-tolerant tetrad forming cocci. Meantime the existing nomenclature is used.

Differentiation from Other Closely Related Taxa

Lactobacilli are metabolically very similar to the other genera of the so-called lactic acid bacteria. Only their rod shape readily distinguishes them from the coccal genera *Streptococcus, Leuconostoc* and *Pediococcus*. However, some species of the obligately heterofermentative lactobacilli form coccoid rods and may be confused with *Leuconostoc*. These species are differentiated from *Leuconostoc* by their formation of DL-lactic acid and not D(−)-lactic acid.

Strains of *Streptococcus* which form atypically elongated cells may also be confused with coccoid rods of lactobacilli. Here, differentiation may require nucleic acid hybridization as in the case of *L. xylosus* and "*L. hordniae*," both of which have been shown to belong to the genus *Streptococcus* (Garvie et al., 1981; Kilpper-Bälz et al., 1982).

The rod-shaped bifidobacteria, which until the eighth edition of *Bergey's Manual* had long been included in the genus *Lactobacillus* as "*Lactobacillus bifidus*," may be differentiated from lactobacilli on the basis of their characteristic hexose fermentation pathway which yields lactic acid and acetic acid at a molar ratio of 2:3, but no CO_2, instead of lactic acid, acetic acid (or ethanol) and CO_2 at a molar ratio of 1:1:1, the pattern of fermentation products typical of obligately heterofermentative lactobacilli.

Taxonomic Comments

The species of the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* form a supercluster within the so-called clostridia subbranch of the Gram-positive bacteria, as shown by oligonucleotide cataloging of their 16S rRNA (Fig. 14.4; Stackebrandt et al., 1983). Bifidobacteria, already excluded from the family *Lactobacillaceae* in *Bergey's Manual*, eighth edition, have proved to be completely unrelated to lactobacilli. They belong to the so-called actinomycetales subbranch of the Gram-positive bacteria.

The neighborhood of the lactobacillus supercluster and the streptococcus cluster, and their position at the clostridia subbranch which also contains the aerobic bacilli (Fig. 14.4) is in accordance with Orla-Jensen's concept of "lactic acid bacteria" as a group of closely related microaerophilic genera. However, there is only limited agreement between the results obtained by oligonucleotide cataloging and the phylogenetic implications of serological studies involving antisera against malic enzymes (London, 1971), fructose-1,6-diphosphate aldolases (London and Kline, 1973; London and Chace, 1976) and glyceraldehyde-3-phosphate dehydrogenases (London and Chace, 1983) of various lactic acid bacteria and some anaerobic and aerobic bacteria. On the basis of the two techniques, only the very close interrelationship between the four genera of lactic acid bacteria and their origin from a common progenitor is certain. Different results were obtained not only regarding the relationship between the lactic acid bacteria and other phylogenetically more distant genera (*Eubacterium, Propionibacterium, Brochothrix, Acholeplasma, Aerococcus*) but also regarding the relationship within the lactic acid bacteria. The immunological grouping indicates a close relationship between streptococci and the *L. casei* group (London and Chace, 1983), whereas, on the basis of the 16S rRNA cataloging, only representatives of the genus *Streptococcus*, but not members of the genera *Pediococcus* and *Leuconostoc*, can be separated

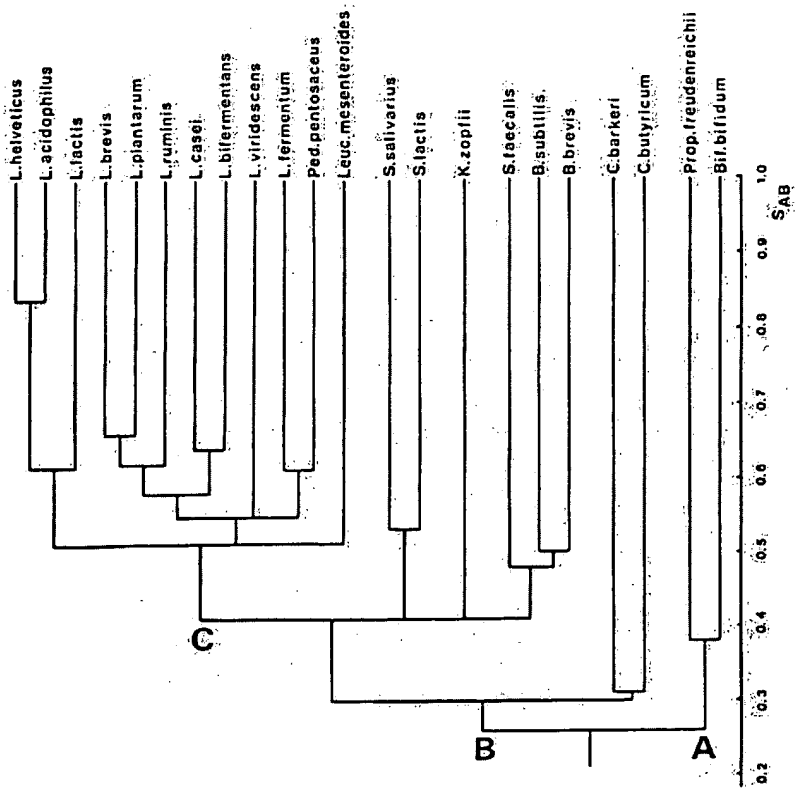

Figure 14.4. Dendrogram of relationship among representatives of the genera *Lactobacillus, Leuconostoc, Pediococcus, Streptococcus, Kurthia, Clostridium, Propionobacterium, Bifidobacterium* and *Bacillus* based on S_{AB} values (16S rRNA cataloging; Stackebrandt et al., 1983). *A*, actinomycetales subbranch; *B*, clostridia subbranch; and *C*, lactobacillus supercluster.

swollen ends. After prolonged incubation, they usually fragment into diphtheroidal or coccoid elements of varied size and shape.

The cell wall contains glucosamine, muramic acid, alanine, glutamic acid, lysine, ornithine, and aspartic acid. Rhamnose is the predominant cell wall sugar, but glucose and fucose may be present in trace amounts.

Microcolonies on agar media and initial growth in liquid media are usually filamentous. Mature colonies are small, opaque, smooth, entire, convex, with a dark central region. Rough colony variants occur occasionally. Pigmentation is not evident. In liquid media, growth is granular or flocculent forming a white sediment without turbidity.

The optimum growth temperature is approximately 30°C; poor or no growth at 37°C.

The organism does not grow on media lacking organic nitrogen. In addition, little if any growth is obtained in certain chemically defined media or media containing simple peptones.

Other descriptive and differential characteristics are listed in Tables 15.47 and 15.49.

Using the fluorescent antibody technique, no cross-reactivity was observed between "*A. humiferus*" and other *Actinomyces* or *Rothia* species. A slight cross-staining obtained with *Corynebacterium (Bacterionema) matruchotii* antiserum was considered nonspecific.

The natural habitat of "*A. humiferus*" appears to be organically rich soil from which the organism may be recovered in high numbers. Experimental infection could not be induced in mice after intraperitoneal injection of washed saline cell suspensions.

The mol% G + C of the DNA is 73 on average (density gradient).

Type strain: ATCC 25174.

Genus Bifidobacterium *Orla-Jensen 1924, 472*[AL]

VITTORIO SCARDOVI

Bi.fi.do.bac.te'ri.um. L. adj. *bifidus* cleft, divided; Gr. dim. n. *bakterion* a small rod; M.L. neut. n. *Bifidobacterium* a cleft rodlet.

Rods of various shapes: short, regular, thin cells with pointed ends; coccoidal regular cells, long cells with slight bends or protuberances or with a large variety of branchings; pointed, slightly bifurcated club-shaped or spatulated extremities; single or in chains of many elements; in star-like aggregates or disposed in "V" or "palisade" arrangements. Colonies smooth, convex, entire edges, cream to white, glistening and of soft consistency. Gram-positive, non-acid-fast; nonspore-forming, nonmotile. Cells often stain irregularly with methylene blue. Anaerobic; some species can tolerate O_2 only in the presence of CO_2. Optimum growth temperature 37–41°C; minimum growth temperatures 25–28°C maximum 43–45°C. Optimum pH for initial growth 6.5–7.0; no growth at 4.5—5.0 or 8.0–8.5

Saccharoclastic. Acetic and lactic acid are formed primarily in the molar ratio of 3:2. CO_2 is not produced (except in the degradation of gluconate). Small amounts of formic acid, ethanol and succinic acid are produced. **Butyric and propionic acid are not produced. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate shunt** in which fructose-6-phosphoketolase (F6PPK-EC 4.1.2.22) cleaves fructose-6-phosphate into acetylphosphate and erythrose-4-phosphate. End products are formed through the sequential action of transaldolase (EC 2.2.1.2), transketolase (EC 2.2.1.1), xylulose-5-phosphate phosphoketolase (EC 4.1.2.9) and enzymes of EMP acting on glyceraldehyde-3-phosphate. Additional acetic and formic acid may be formed through a cleavage of pyruvate.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49, $NADP^+$- or NAD^+-dependent) generally not determinable.

Catalase-negative except that *B. indicum* and *B. asteroides* are catalase-positive when grown in the presence of air with or without added hemin.

Ammonium is generally utilized as a source of nitrogen.

The G + C content of DNA (Bd or T_m) varies from 55–67 mol%.

The organisms occur in the intestine of man, various animals and honey bees; found also in sewage and human clinical material.

Type species: *Bifidobacterium bifidum* (Tissier) Orla-Jensen 1924, 472.

Further Descriptive Information

Morphology

The cellular morphology and its variations, as affected by different cultural conditions, have been widely investigated (see Poupard, Husain and Norris, 1973, for references). However, recent discoveries of new species from a variety of habitats have permitted a clearer picture of the morphology of the genus.

A comparison of the cell morphology of large numbers of strains grown anaerobically (GasPak system, BBL) in stabs of trypticase-phytone-yeast extract medium (TPY) showed that some species had distinct cell shapes or arrangements which might be of help in their recognition; these traits are reported in Figs. 15.96–15.98.

Outstanding are the well known amphora-like cells of *B. bifidum* (Sundman et al., 1959) (Fig. 15.96*A*), the V or palisade arrangement of cells in *B. angulatum* (Fig. 15.96*D*), the linear groups of globular elements in *B. catenulatum* (Fig. 15.96*E*), the long chains of regular cells in *B. pullorum* (Fig. 15.97*A*), the middle-enlarged cells of *B. animalis* (Fig. 15.97*B*), the large cellular dimensions in *B. magnum* (Fig. 15.97*D*), the small cells of *B. minimum* (Fig. 15.97*F*), and the unusual starlike arrangements of cells in *B. asteroides* (Fig. 15.98*A*). The cellular shape most frequently encountered in those species not having distinct morphology (see Table 15.50) as observed in TPY stabs (see above), is depicted in Fig. 15.98*D*. Details are given under single species description.

B. asteroides (starlike clusters) and *B. indicum* (small rods or coccobacilli), the species with the most nonbifid-like morphology in the classic sense, show features common to the morphology of the other bifids only when grown in nutritionally deficient media (Scardovi and Trovatelli, 1969), which seems to be a general trend in this group of bacteria (Sundman and Björksten, 1958; Glick et al., 1960).

Cell Wall Structure

The most extensive study of cell wall murein structure of bifidobacteria has been made by O. Kandler and collaborators (reported later in Table 15.50). Closely related species can be clearly distinguished on this basis, i.e. *B. boum* from *B. thermophilum* or *B. minimum* from *B. subtile*.

On the basis of murein structure, bifidobacteria are more closely related to *Lactobacillaceae* than to *Actinomycetaceae* (Kandler and Lauer, 1974).

Lipid Cellular Composition

Some species of *Bifidobacterium* and *Lactobacillus* were studied by Exterkate et al. (1971): differences in polyglycerol phospholipids and aminoacyl phosphatidylglycerol were found to be of help in differentiating the two genera. The effects of growth conditions on the lipid and ionic composition of *B. bifidum* subsp. *pennsylvanicum* have been recently studied by Veerkamp (1977a, b).

Ultrastructure

The ultrastructure of bifidobacteria has received little attention. Overman and Pine (1963) first reported ultrastructure micrographs of *B. bifidum* subsp. *pennsylvanicum*. Recently, Zani and Severi (1982)

BALNEATRIX 43:624
Balneatrix alpica 43:624

BALNEIMONAS 54:631
Balneimonas flocculans (corrig.) 54:631

BARTONELLA
Bartonella alsatica 49:287*
Bartonella bacilliformis
Bartonella birtlesii 50:1978*
Bartonella bovis 52:388*
Bartonella capreoli 52:388*
Bartonella chomelii 54:219*
Bartonella clarridgeiae 46:836
Bartonella doshiae 45:7*
Bartonella elizabethae 43:785*
Bartonella grahamii 45:7*
Bartonella henselae 43:785*
Bartonella koehlerae 50:423
Bartonella peromysci 45:7*
Bartonella quintana 43:784*
Bartonella schoenbuchensis (corrig.) 51:1563*
Bartonella talpae 45:7*
Bartonella taylorii 45:7*
Bartonella tribocorum 48:1338*
Bartonella vinsonii see: *B. vinsonii* subsp. *vinsonii*
Bartonella vinsonii subsp. *arupensis* 50:3
Bartonella vinsonii subsp. *berkhoffii* 46:708*
Bartonella vinsonii subsp. *vinsonii* 43:785*

BDELLOVIBRIO
Bdellovibrio bacteriovorus
Bdellovibrio starrii → *Bacteriovorax starrii* → *Peredibacter starrii*
Bdellovibrio stolpii → *Bacteriovorax*

BEGGIATOA
Beggiatoa alba

BEIJERINCKIA
Beijerinckia derxii see: *B. derxii* subsp. *derxii*
Beijerinckia derxii subsp. *derxii*
Beijerinckia derxii subsp. *venezuelae* 31:215
Beijerinckia fluminensis
Beijerinckia indica see: *B. indica* subsp. *indica*
Beijerinckia indica subsp. *indica* 31:215
Beijerinckia indica subsp. *lacticogenes* 31:215
Beijerinckia mobilis

BELLIELLA 54:69*
Belliella baltica 54:69*

BELNAPIA 56:56*
Belnapia moabensis 56:57*

BENECKEA → VIBRIO
Beneckea alginolytica = *Vibrio alginolyticus*
Beneckea campbellii → *Vibrio*
Beneckea gazogenes → *Vibrio*
Beneckea harveyi → *Vibrio*
Beneckea natriegens → *Vibrio*
Beneckea nereis (corrig.) → *Vibrio*
Beneckea nigripulchritudo (corrig.) → *Vibrio*
Beneckea parahaemolytica = *Vibrio parahaemolyticus*
Beneckea pelagia → *Vibrio pelagius* → *Listonella pelagia*
Beneckea splendida → *Vibrio splendidus*
Beneckea vulnifica → *Vibrio vulnificus*

BERGERIELLA 55:1395
Bergeriella denitrificans 55:1395

BERGEYELLA 44:830*
Bergeyella zoohelcum 44:830*

BEUTENBERGIA 49:1738*
Beutenbergia cavernae 49:1738*

BIFIDOBACTERIUM
Bifidobacterium adolescentis
Bifidobacterium angulatum
Bifidobacterium animalis see: *B. animalis* subsp. *animalis*
Bifidobacterium animalis subsp. *animalis*
Bifidobacterium animalis subsp. *lactis* 54:1142*
Bifidobacterium asteroides
Bifidobacterium bifidum
Bifidobacterium boum
Bifidobacterium breve
Bifidobacterium catenulatum
Bifidobacterium choerinum
Bifidobacterium coryneforme 32:368*
Bifidobacterium cuniculi
Bifidobacterium denticolens → *Parascardovia*
Bifidobacterium dentium
Bifidobacterium gallicum 40:100*
Bifidobacterium gallinarum 33:127*
Bifidobacterium globosum → *B. pseudolongum* subsp. *globosum*
Bifidobacterium indicum
Bifidobacterium infantis ⇒ *B. longum*
Bifidobacterium inopinatum → *Scardovia inopinata*
Bifidobacterium lactis → *Bifidobacterium animalis* - *B. animalis* subsp. *lactis*
Bifidobacterium longum
Bifidobacterium magnum
Bifidobacterium merycicum 41:167*
Bifidobacterium minimum 32:368*
Bifidobacterium pseudocatenulatum
Bifidobacterium pseudolongum see: *B. pseudolongum* subsp. *pseudolongum*
Bifidobacterium pseudolongum subsp. *globosum* 42:656
Bifidobacterium pseudolongum subsp. *pseudolongum* 42:656
Bifidobacterium psychraerophilum 54:405*
Bifidobacterium pullorum
Bifidobacterium ruminantium 41:165*
Bifidobacterium saeculare 42:191
Bifidobacterium scardovii 52:998*
Bifidobacterium subtile 32:368*
Bifidobacterium suis ⇒ *B. longum*
Bifidobacterium thermacidophilum see: *B. thermacidophilum* subsp. *thermacidophilum*
Bifidobacterium thermacidophilum subsp. *porcinum* 53:1622*
Bifidobacterium thermacidophilum subsp. *thermacidophilum* 50:124*; 53:1622*
Bifidobacterium thermophilum

BILOPHILA 40:320
Bilophila wadsworthia 40:320

Kitasatoa nagasakiensis ⇒ *Streptomyces purpureus*
Kitasatoa purpurea → *Streptomyces purpureus*

KITASATOSPORA (corrig.) = ***STREPTOMYCES***
Kitasatospora arboriphila 54:2127*
Kitasatospora azatica (corrig.) 47:1053*
Kitasatospora cheerisanensis 49:757*
Kitasatospora cineracea 51:1770*
Kitasatospora cochleata (corrig.) 47:1053*
Kitasatospora cystarginea (corrig.) = *Streptomyces cystargineus*
Kitasatospora gansuensis 54:2127
Kitasatospora griseola (corrig.) = *Streptomyces griseolosporeus*
Kitasatospora kifunensis 53:2038*
Kitasatospora mediocidica (corrig.) = *Streptomyces mediocidicus*
Kitasatospora niigatensis 51:1770*
Kitasatospora nipponensis 54:2127
Kitasatospora paracochleata (corrig.) 47:1053*
Kitasatospora paranensis 54:2128*
Kitasatospora phosalacinea (corrig.) = *Streptomyces phosalacineus*
Kitasatospora putterlickiae 53:2037*
Kitasatospora sampliensis 56:522*
Kitasatospora setae (corrig.) = *Streptomyces*
Kitasatospora terrestris 54:2128
Kitasatospora viridis 55:710*

KLEBSIELLA
Klebsiella granulomatis 49:1698*
Klebsiella mobilis = *Enterobacter aerogenes*
Klebsiella ornithinolytica → *Raoultella*
Klebsiella oxytoca
Klebsiella ozaenae → *K. pneumoniae* subsp. *ozaenae*
Klebsiella planticola → *Raoultella*
Klebsiella pneumoniae
Klebsiella pneumoniae subsp. *ozaenae* 34:355
Klebsiella pneumoniae subsp. *pneumoniae* 34:355
Klebsiella pneumoniae subsp. *rhinoscleromatis* 34:355
Klebsiella rhinoscleromatis → *K. pneumoniae* subsp. *rhinoscleromatis*
Klebsiella singaporensis 54:2135*
Klebsiella terrigena → *Raoultella*
Klebsiella trevisanii ⇒ *Klebsiella planticola* → *Raoultella planticola*
Klebsiella variicola 54:631

KLUYVERA 31:382
Kluyvera ascorbata 31:382
Kluyvera cochleae ⇒ *K. intermedia*
Kluyvera cryocrescens 31:382
Kluyvera georgiana 46:63*
Kluyvera intermedia 55:441*

KNOELLIA 52:81*
Knoellia sinensis 52:82*
Knoellia subterranea 52:82*

KOCURIA 45:690*
Kocuria carniphila 55:141*
Kocuria erythromyxa ⇒ *K. rosea*
Kocuria kristinae 45:690*
Kocuria marina 54:1619*
Kocuria palustris 49:171*
Kocuria polaris 53:187*
Kocuria rhizophila 49:172*
Kocuria rosea 45:690*
Kocuria varians 45:690*

KORDIA 54:678*
Kordia algicida 54:679*

KORDIIMONAS 55:2036*
Kordiimonas gwangyangensis 55:2037*

KOSERELLA ⇒ ***YOKENELLA***
Koserella trabulsii ⇒ *Yokenella regensburgei*

KOZAKIA 52:816*
Kozakia baliensis 52:817*

KRIBBELLA 49:750*
Kribbella antibiotica 54:1425
Kribbella flavida 49:750*
Kribbella jejuensis 54:1348*
Kribbella koreensis 53:1007*
Kribbella lupini 56:410*
Kribbella sandramycini 49:750*
Kribbella solani 54:1347*

KROKINOBACTER 56:326*
Krokinobacter diaphorus 56:327*
Krokinobacter eikastus 56:327*
Krokinobacter genikus 56:327*

KURTHIA
Kurthia gibsonii 33:672
Kurthia sibirica 38:220
Kurthia zopfii

KUTZNERIA 44:267*
Kutzneria albida 44:268*
Kutzneria kofuensis 44:268*
Kutzneria viridogrisea 44:268*

KYTOCOCCUS 45:687*
Kytococcus schroeteri 52:1613*
Kytococcus sedentarius 45:687*

LABRYS 35:375
Labrys methylaminiphilus 55:1252*
Labrys monachus 35:375

LACEYELLA 55:398*
Laceyella putida 55:399*
Laceyella sacchari 55:399*

LACHNOBACTERIUM 51:1980*
Lachnobacterium bovis 51:1980*

LACHNOSPIRA
Lachnospira multipara
Lachnospira pectinoschiza 44:92*

LACINUTRIX 55:1482*
Lacinutrix copepodicola 55:1482*

LACTOBACILLUS
Lactobacillus acetotolerans 36:544*
Lactobacillus acidifarinae 55:619*
Lactobacillus acidipiscis 50:1481*
Lactobacillus acidophilus
Lactobacillus agilis 32:266
Lactobacillus algidus 50:1148*

Lactobacillus alimentarius 33:672
Lactobacillus amylolyticus 49:1
Lactobacillus amylophilus 31:216
Lactobacillus amylovorus 31:61*
Lactobacillus animalis 33:438
Lactobacillus antri 55:81*
Lactobacillus arizonensis ⇒ *L. plantarum*
Lactobacillus aviarius 35:223
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Streptococcus lactis → *Lactococcus lactis* subsp. *lactis*
Streptococcus lactis subsp. *cremoris* → *Lactococcus lactis* subsp. *cremoris*
Streptococcus lactis subsp. *diacetilactis* ⇒ *Lactococcus lactis* subsp. *lactis*
Streptococcus lutetiensis 52:1253*
Streptococcus macacae 34:333*
Streptococcus macedonicus → *S. gallolyticus* subsp. *macedonicus*
Streptococcus marimammalium 55:274*
Streptococcus minor 54:451*
Streptococcus mitis
Streptococcus morbillorum → *Gemella*
Streptococcus mutans
Streptococcus oligofermentans 53:1103*
Streptococcus oralis 32:410*
Streptococcus orisratti 50:60*
Streptococcus ovis 51:1149*
Streptococcus parasanguinis (corrig.) 40:321
Streptococcus parauberis 40:470
Streptococcus parvulus → *Atopobium parvulum*
Streptococcus pasteurianus → *S. gallolyticus* subsp. *pasteurianus*
Streptococcus peroris 48:926*
Streptococcus phocae 44:649*
Streptococcus plantarum → *Lactococcus*
Streptococcus pleomorphus
Streptococcus pluranimalium 49:1225*
Streptococcus pneumoniae
Streptococcus porcinus 35:224
Streptococcus pseudopneumoniae 55:1
Streptococcus pyogenes
Streptococcus raffinolactis → *Lactococcus*
Streptococcus ratti (corrig.)
Streptococcus saccharolyticus → *Enterococcus*
Streptococcus salivarius
Streptococcus salivarius subsp. *thermophilus* → *S. thermophilus*
Streptococcus sanguinis (corrig.)
Streptococcus shiloi ⇒ *S. iniae*
Streptococcus sinensis 52:1438
Streptococcus sobrinus 33:883*
Streptococcus suis 37:160*
Streptococcus thermophilus 45:619
Streptococcus thermophilus → *Streptococcus salivarius* subsp. *thermophilus* → *S. thermophilus*
Streptococcus thoraltensis 47:1077*
Streptococcus uberis
Streptococcus urinalis 50:1177*
Streptococcus vestibularis 38:335*
Streptococcus waius ⇒ *Streptococcus macedonicus* → *S. gallolyticus* subsp. *macedonicus*

STREPTOMONOSPORA (corrig.) 51:362*
Streptomonospora alba 53:1424*
Streptomonospora salina (corrig.) 51:362*

STREPTOMYCES
Streptomyces abikoensis 41:456
Streptomyces aburaviensis
Streptomyces achromogenes see: *S. achromogenes* subsp. *achromogenes*
Streptomyces achromogenes subsp. *achromogenes*
Streptomyces achromogenes subsp. *rubradiris*
Streptomyces acidiscabies 39:393*
Streptomyces acrimycini
Streptomyces aculeolatus 38:136
Streptomyces afghaniensis
Streptomyces africanus 54:1534*
Streptomyces alanosinicus
Streptomyces albaduncus
Streptomyces albiaxialis 43:398
Streptomyces albidochromogenes 36:573
Streptomyces albidoflavus
Streptomyces albireticuli ⇒ *S. eurocidicus*
Streptomyces albofaciens
Streptomyces alboflavus
Streptomyces albogriseolus
Streptomyces albolongus
Streptomyces alboniger
Streptomyces albospinus
Streptomyces albosporeus see: *S. albosporeus* subsp. *albosporeus*
Streptomyces albosporeus subsp. *albosporeus* ⇒ *S. aurantiacus*
Streptomyces albosporeus subsp. *labilomyceticus*
Streptomyces alboverticillatus ⇒ *S. griseocarneus*
Streptomyces albovinaceus
Streptomyces alboviridis
Streptomyces albulus
Streptomyces albus see: *S. albus* subsp. *albus*

Johansen, 1996), and cheeses produced with these strains have better flavour than those produced with an identical strain lacking the lysin gene. Presumably, the improved flavour results from the lysis of cells in the cheese matrix, allowing the release of intracellular peptidases.

In order to obtain the maximum benefit from traditional and molecular genetics, it is necessary to understand the metabolic processes of LAB which are relevant for cheesemaking. Some of these are discussed below.

4.8 Biochemistry of acidification by lactic acid bacteria

The long historical application of LAB in the production of a wide range of fermented food products is primarily due to the high capacity of the LAB to produce lactic acid and, thereby, preserve the food products. The production of lactic acid by LAB not only serves as a competitive advantage when growing in their natural habitats but also leads to the generation of metabolic energy, in the form of adenosine triphosphate (ATP), which is required for growth and proliferation.

There have been several excellent reviews on the acidification biochemistry and regulation of sugar metabolism in LAB (for example, Yamada, 1987; Monnet *et al.*, 1996; and Cocaign-Bousquet *et al.*, 1996).

4.8.1 Lactose metabolism

When LAB grow in milk, lactose is converted to lactic acid, and sometimes, acetate, ethanol and carbon dioxide. Lactose is converted by a series of enzymatic reactions into either L(+)- or D(−)-lactate, or a mixture of the two (Table 4.3). The isomeric mixture of lactate has several important aspects. One example is in the manufacture of Swiss-type cheeses, where *Propionibacterium* preferentially metabolises and grows faster on the L-isomer. *Lc. lactis* and *S. thermophilus* exclusively produce the L-isomer, while *Leuconos-*

Table 4.3 Lactate production by various lactic acid bacteria (LAB)

Organism	Transport system	Pathway	Main fermentation products[a]	Lactate isomer
Lactococcus	PEP-PTS	Homofermentative	4 lactate	L
Streptococcus	Permease	Homofermentative	2 or 4 lactate[b]	L
Lactobacillus	Permease	Homofermentative	2 or 4 lactate[b]	D or DL
Leuconostoc	Permease	Heterofermentative	2 lactate + 2 ethanol + 2 CO_2	D

[a] Moles per mole fermented lactose; [b] 2 moles of lactate if galactose is stoicheiometrically secreted, and 4 moles of lactate if galactose is fully metabolised. Abbreviation: PEP-PTS, phosphoenol pyruvate-dependent phosphotransferase system.

toc produces only the D-isomer. *Lactobacillus* produces either D-lactate (e.g. *Lb. delbrueckii*) or a mixture of the two isomers (e.g. *Lb. helveticus*).

Lactose is transported across the cellular membrane and into the cytoplasm either by the phosphoenol pyruvate-dependent phosphotransferase system (PEP-PTS) or by a lactose permease. The PEP-PTS system consists of a sequence of enzymatic phosphorylation/dephosphorylation reactions, by which lactose is transported and phosphorylated to lactose phosphate at the expense of phosphoenol pyruvate, which is converted to pyruvate. Lactose phosphate is hydrolysed to glucose and galactose-6-phosphate by phospho-β-galactosidase. In strains with a lactose permease, lactose is transported without modification and hydrolysed to glucose and galactose by β-galactosidase. Galactose is then phosphorylated and converted to glucose-6-phosphate by the Leloir pathway. In *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis*, galactose is excreted into the medium. A sequential sugar metabolism is often observed in mixed cultures; galactose is only metabolised when lactose becomes limiting. Uptake of galactose takes place either via a permease or a PEP-PTS, similar to the uptake of lactose, depending on the species. Galactose-6-phosphate is converted into triose phosphates via the tagatose pathway and enters the homofermentative pathway.

The glucose moiety of lactose is phosphorylated to glucose-6-phosphate, which in *Lactococcus*, *Streptococcus* and *Lactobacillus* is further catabolised to lactate by the homofermentative pathway, whereas *Leuconostoc* catabolises glucose-6-phosphate by the heterofermentative pathway to lactate, ethanol and carbon dioxide.

4.9 Proteolysis by lactic acid bacteria

Proteolysis results in textural changes in the cheese, by disruption of the protein matrix, and in flavour development, due to the release of small peptides and free amino acids. In addition to the coagulant and plasmin, starter LAB make a significant contribution to cheese proteolysis. The proteolytic system of *Lc. lactis* consists of a cell-envelope associated proteinase, known as lactocepin, and 12–15 different intracellular peptidases. There are no reliable reports of extracellular peptidases or proteinases in *Lc. lactis* (Tan *et al.*, 1993).

Based on specificity differences in the hydrolysis of the caseins by lactocepin, three different types of proteinase have been recognised, i.e. types I, III and I/III. Lactocepin type I hydrolyses β-casein and κ-casein but not α_{s1}-casein; type III hydrolyses β-casein, κ-casein and α_{s1}-casein; and type I/III has a mixture of type I and type III specificity (Visser, 1993). However, more recently, the classification of lactocepin has been further refined, based

5

Separation of Species of the Genus *Leuconostoc* and Differentiation of the Leuconostocs from Other Lactic Acid Bacteria

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I. Introduction

The genus *Leuconostoc* comprises six species (Skerman *et al.*, 1980) but there is evidence that these should be reduced to four (Section IV). If this is accepted both *Leuconostoc dextranicum* and *L. cremoris* will be reduced to subspecies of *L. mesenteroides* (Garvie, 1983). In this chapter, the current names are used, but the evidence for the proposed changes in classification is given.

Leuconostocs are normally found living in association with vegetable matter with lactose-fermenting species occurring in milk and dairy products. Leuconostocs are not pathogenic to plants or animals. The genus is of considerable commercial importance with *L. paramesenteroides* as the only species without commercial significance. In nature, leuconostocs are occasionally the dominant flora, but usually they occur as minor components of a bacterial population and are either not recognized or are overlooked. Certain strains are now cultured for commercial reasons with the main emphasis on the particular chemical reaction for which they are important, and with less emphasis on identification and classification of strains.

All lactic acid bacteria depend on the fermentation of carbohydrates for energy, and all form lactate as a major end-product of fermentation of glucose. They can be divided into homofermentative and heterofermentative species depending on the end-products from the fermentation of glucose when they are growing in a good nutritive medium. The homofermentative species use the Embden-Myerhof (EM) glycolytic pathway (Fig. 1) converting glucose to fructose-1,6-diphosphate (EDP), which is split to glyceraldehyde phosphate. The end-product is two moles of lactic acid for each mole of glucose consumed. Fructose-1,6-diphosphate aldolase is a key enzyme in the EM pathway. Streptococci, pediococci and many lactobacilli are homofermentative. Some species possess alternative glycolytic pathways which will be used when, for some reason, the EM pathway is suppressed. The heterofermentative lactic acid bacteria comprise leuconostocs and some lactobacilli; fructose-1,6-diphosphate aldolase is absent and the EM pathway is not used. Instead glucose is converted to glucose-6-phosphate and then to 6-phosphogluconate which is decarboxylated. The resulting pentose is converted to lactic acid and ethanol and/or acetate (Blackwood and Blakley, 1960). This fermentation follows the pentose phosphate pathway initially and then the phosphoketolase pathway. Glucose-6-phosphate dehydrogenase and xylulose-5-phosphate phosphoketolase are key enzymes. The former has a cheap substrate and is easy to assay, the latter has an expensive substrate or can be assayed starting from ribose-5-phosphate, which is not always satisfactory. The production of CO_2 from glucose is easily assessed and, often, a very obvious property of heterofermentative lactic acid bacteria.

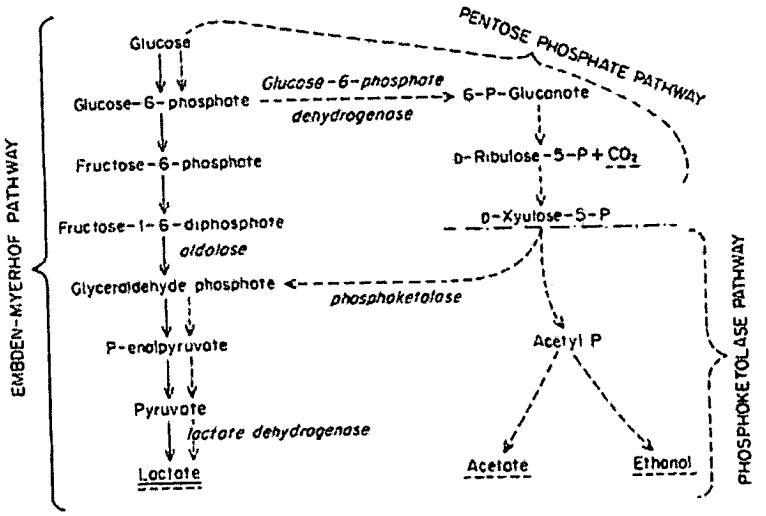

Fig. 1. Glycolytic pathways in lactic acid bacteria. Main pathway in homofermentative lactic acid bacteria ——. Main pathway in heterofermentative lactic acid bacteria – – –.

The differences between the homofermentative and heterofermentative bacteria are unrelated to morphology which is still used as a primary method of classification. In many ways the leuconostocs have more in common with the heterofermentative lactobacilli than with the streptococci. This will be discussed further in later sections.

II. Isolation of leuconostocs and general media

A. Isolation from natural habitats

Selective media specific for leuconostocs have not been devised and cultures are isolated from mixed populations because they grow under the conditions chosen for the selection of some other lactic acid bacteria. It is difficult to obtain an estimation of leuconostocs in mixed populations without isolating and identifying randomly picked colonies. Cavett *et al.* (1965) used a combination of 1.0% thallous acetate and 0.01% 2,3,5-triphenyltetrazolium chloride added to a peptone meat extract sucrose medium to distinguish *Streptococcus lactis* and *S. faecalis* from leuconostocs. 5.0% sucrose was included so that colonies forming dextran could be counted, these were grouped as leuconostocs. This procedure is not satisfactory as it could

include dextran forming lactobacilli and would exclude non-dextran forming leuconostocs.

Leuconostocs live mainly on vegetable matter where Gram-negative organisms and aerobic spore formers may predominate and are likely to outgrow any leuconostoc present. When surface scrapings of vegetables are inoculated into a yeast glucose phosphate peptone broth (YGPB or Medium 1, Appendix I) containing 0.1% thallium acetate and 0.0005% crystal violet and incubated until turbid, the spore formers and Gram-negative rods are suppressed. Subsequent plating on agar medium of the same composition as the broth allows isolation of Gram-positive cocci. This technique does not enable any estimation of the initial population of leuconostocs to be made. Both *L. mesenteroides* and *L. paramesenteroides* will grow on acetate agar selective for lactobacilli. It is probable that *L. dextranicum* and *L. lactis* will also grow, but unlikely that *L. cremoris* would form colonies as it would be outgrown by any lactobacilli or other leuconostocs present. The pH (5.4) of the acetate agar would also act as an inhibitor of *L. cremoris*.

In a mixed population containing known species, i.e. cheese and butter starter, it is possible to monitor the numbers of each species present. Table I shows the properties which distinguish leuconostocs from starter streptococci. Leuconostocs are more fastidious and require a more complex growth medium than streptococci. The combination of the ability to use citrate and to hydrolyse arginine can be used to recognize the components of starter culture. No lactic acid bacteria use citrate as a source of energy but can break it down to form important flavour components when cultures are growing with a carbohydrate to supply energy. The breakdown of citrate results in the production of acetoin/diacetyl, i.e.

$$\begin{array}{l} \text{Citrate} \rightarrow \text{oxaloacetate} + \text{acetate} \\ \qquad\qquad \downarrow \\ \qquad CO_2 + \text{pyruvate} \\ \qquad\qquad\quad \downarrow \\ \qquad\qquad\quad \text{Acetoin/diacetyl} + CO_2 \end{array}$$

The pyruvate from all sources are pooled and the excess over that required for the regeneration of NADH is converted to acetoin. Galesloot *et al.* (1961) describe the preparation and use of a medium containing insoluble calcium citrate which can be suspended in carboxymethyl cellulose and then this suspension incorporated into agar media. The medium is opaque, but citrate-utilizing colonies form clear zones. The carbohydrate content of the medium is low, which both encourages the bacteria to use citrate and prevents acid formed by non-citrate users dissolving the calcium and causing clear zones. This medium is most useful when the components of the bacterial mixture are known, for example cheese starter cultures where any leuconostocs present

TABLE I

Some differentiating properties of cheese starter streptococci and leuconostocs

	Streptococci			Leuconostocs	
	S. lactis	*S. lactis* subsp. *diacetylactis*	*S. cremoris*	*L. lactis*	*L. cremoris*
Utilization of citrate	−	+	−	+*	+
Growth on whey agar	+	+	+	−	−
Growth on supplemented whey agar	+	+	+	+	+
Arginine hydrolysed	+	Usually +	−	−	−

*Not all strains use citrate, but those that do not are unlikely to be a component of starters.

will be citrate users. Not all leuconostocs use citrate and some which do may fail to grow on Galesloot's medium. The agar has limited uses with wild populations. *S. lactis* subsp. *diacetylactis* is common in cheese starter cultures and uses citrate. It normally hydrolyses arginine so that agar media with a low glucose content but containing 1.5% L-arginine will become alkaline while leuconostocs will form acid in the zone around each colony. The inclusion of both arginine and citrate is useful in separating leuconostocs from *S. lactis* subsp. *diacetylactis*.

In wine, leuconostocs and lactobacilli coexist. They can be separated from acetobacter, which will not grow under anaerobic conditions, and from spore formers which are inhibited at low pH (4.5). Yeasts will be inhibited when actidione (20 mg l^{-1}) is added to media. Leuconostocs and lactobacilli have similar growth requirements and only the isolation of colonies followed by identification will show which bacteria are present. Most workers favour a grape juice/yeast extract agar with or without added organic acids (Peynaud and Dupuy, 1964). Tomato juice can be used in place of, or in addition to, grape juice; ATB (Table I in Appendix I) is given as a medium suitable for the growth of *L. oenos*. Leuconostocs living in wine prefer a low growth temperature (20–25°C), an atmosphere containing 10% CO_2, and it can take up to seven to ten days for visible colonies to develop.

B. General method of cultivation

1. *Media*

L. oenos lives only in wine and associated environments not inhabited by other species of the genus. Different media and growth conditions are used

for *L. oenos* from those for other species and, even when identification tests are done, media are adapted to suit acidophilic or non-acidophilic strains.

The composition of some general media are given in Table I in Appendix I. It has been found that MRS is suitable for most leuconostocs and pediococci, as well as the lactobacilli for which it was designed. MRS is not normally used for streptococci which can grow on less complex media, which only support poor growth of leuconostocs, even of the least fastidious species *L. mesenteroides*. MRS contains 2% glucose but this is not necessary for leuconostocs which cease growing at about pH 4.5, in contrast to lactobacilli and pediococci which lower the pH to 4.0 or less. The high sugar content in MRS can cause charring in autoclaving, particularly in large volumes (500 ml and over). When large volumes of any media are required it is suggested that the glucose (or other carbohydrates) is autoclaved (15 lb, 15 min) separately as an aqueous solution and added aseptically to the rest of the medium before inoculation. When Medium 2 was prepared the glucose and cysteine were dissolved in the tomato juice, Seitz filtered and added to the rest of the medium which had been autoclaved (15 lb, 15 min). In some early work a yeast glucose citrate broth (YGCB) was used but this has now been replaced by Medium 1, YGPB or MRS.

2. *Growth conditions*

Most leuconostocs grow at 30°C (37°C is too high) and *L. cremoris* and *L. oenos* prefer 22°C which is probably a better general temperature for the whole genus, although growth will be slower than at 30°C with many strains. *L. cremoris* and *L. oenos* grow better when 0.05% cysteine is added to the media and cysteine does not inhibit growth of other species. In broth all species will grow under atmospheric conditions but an atmosphere of H_2 + 10% CO_2 is better for colonies growing on an agar surface. At one time it was argued that leuconostocs were more aerobic than other lactic acid bacteria because they metabolized glucose by the hexose monophosphate pathway but reducing conditions have been found to improve the growth of a number of strains which grow poorly aerobically. No leuconostocs grow rapidly but many strains give good growth with overnight incubation; *L. cremoris* and isolated strains of other species may require 48 h incubation while *L. oenos* may require seven to ten days before reasonable turbidity is produced in broth.

III. Identification of species

A. Preliminary identification and separation from other lactic acid bacteria

Table II gives a few easily determined characters which should be adequate for placing any lactic acid bacteria in its correct genus. Morphology may present difficulties because both leuconostocs and streptococci can form ovoid and even rod-shaped cells, but both are usually coccoid when growing in milk or supplemented milk. Lactobacilli, on the other hand, can be short rods with pointed ends, in other words the morphology of these three genera merge and it may be difficult to be sure to which genus a particular strain belongs. Morphology combined with gas production and hydrolysis of arginine should allow for correct identification of leuconostocs, but gas production may be weak in freshly isolated strains. The technique described by Abd-el-Malek and Gibson (1948) is reliable providing a heavy inoculum of a well growing culture is used for inoculation. No leuconostocs hydrolyse

TABLE II
Differentiation of leuconostocs from other lactic acid bacteria

	Leuconostocs	Lactobacilli		Streptococci	Pediococci
		Heterofermentative	Homofermentative		
Morphology	Coccus-cocco-bacillus	Cocco-bacillus →rod	Cocco-bacillus →rod	Coccus→ cocco-bacillus	Coccus
Division	1 plane	1 plane	1 plane	1 plane	2 planes
Growth in litmus milk	−or slight	−	−or slight	Usually good	−or slight
Gas from glucose	+	+	−	−	−
Hydrolysis of arginine	−	−or+	−	−or+	−or+
Dextran from sucrose	−or+	−or+	−	Mostly −, a few species +	−
Type of lactic acid	D(−)	DL	D(−), DL or L(+)	L(+)	DL or L(+)
Habitat Plant	+	+	+	A few species +	+
Animal	−	+	+	+	−

arginine, whereas many species of heterofermentative lactobacilli do. The main exceptions are *Lactobacillus viridescens* and occasional strains of *Lb. confusus* and, as both these species form dextran from sucrose, their separation from leuconostocs may be difficult. The difference and similarities of these two lactobacilli to the leuconostocs are discussed further.

B. Further identification by classical methods

The basis for arguing that the heterofermentative lactic acid bacteria form a natural group has been given in Section I, and the properties of *Lb. confusus* and *Lb. viridescens* will be given along with those of leuconostocs. These two lactobacilli are biochemically closer to the non-acidophilic leuconostocs than to the other heterofermentative lactobacilli. Difficulties in identification have existed in the past but now the similarities between leuconostocs and *Lb. viridescens* and *Lb. confusus* are recognized and the characteristics of each species understood errors in identification should be few. *Lb. viridescens* was first recognized as a species by Niven and Evans in 1957 and *Lb. confusus* by Holzapfel and Kandler in 1969. Several strains of *Lb. confusus* studied by Sharpe *et al.* (1972) had been identified as *L. mesenteroides*, and it is possible that other strains have also been identified as leuconostocs.

1. *General points*

Table III gives the properties of two species of lactobacilli and those of all species of leuconostocs. *L. oenos* is the most easily recognized species because it grows in media with a low initial pH, and it is tolerant of ethanol. Some strains of *L. oenos* have a requirement for 4-0-(α-D-glucopyranosyl)-D-pantothenic acid (Amachi *et al.*, 1970). This substance is also known as tomato juice factor (TJF) (Garvie and Mabbitt, 1967). The requirement varies with different growth conditions and different strains, so that it cannot be used to identify *L. oenos*. Most strains of *L. oenos* will destroy TJF even if they do not have a high requirement for it, but no other lactic acid bacteria are known to use TJF as a form of pantothenate. The requirement for TJF can be overcome by a high level of pantothenate and also by a heavy inoculation so that media free of tomato juice or of TJF can be used for many purposes. Obviously tomato juice cannot be added to media when fermentation properties are studied, in these tests the basal broth used is simple and a high cell inoculation is used. The basal media used for other species is unsuitable for *L. oenos* but can be modified by lowering the pH to 5.2, including 0.5% agar and using 0.004% bromo-cresol green as indicator (Garvie, 1967b). *L. oenos* shows considerable variation in ability to ferment carbohydrates, which has led some workers to consider that there is more

TABLE III

Differentiation of species within the genus *Leuconostoc* and separation from *Lactobacillus confusus* and *Lactobacillus viridescens*

	Leuconostoc					*Lactobacilli*		
	L. mesenteroides subsp. *mesenteroides*	*L. mesenteroides* subsp. *dextranicum*	*L. mesenteroides* subsp. *cremoris*	*L. paramesenteroides*	*L. lactis*	*L. oenos*	*Lb. confusus*	*Lb. viridescens*
Hydrolysis of arginine[a]	−	−	−	+	+	−	(±)	−
Mucoid colonies on sucrose agar[a]	+	+	−	−	−	−	+	±
Lactic acid formed from glucose[a]	D(−)	D(−)	D(−)	D(−)	D(−)	D(−)	DL	DL
Growth at 45°C	−	−	−	+	+	+	(±)	−
Growth at pH 4.8 in CMB[a]	+	+	+	(±)	+	+	NI	NI
Growth at pH 3.7 in CMB[a]	+	+	+	−	+	+	NI	NI
Growth in 10% ethanol in CMB	+	+	+	−	−	+	NI	NI
Dissimilation of citrate[a]	(+)	(+)	(−)	(+)	(+)	−	±	+
Fermentation of:								
Arabinose[a]	+	+	−	(±)	(±)	±	±	−
Xylose[a]	+	±	−	(±)	−	(±)	+	−
Fructose	+	(±)	−	(±)	(±)	+	+	−
Galactose	(±)	(±)	+	+	+	(±)	+	(±)
Mannose	(±)	+	−	+	(±)	(±)	+	−
Cellobiose	+	(±)	−	±	−	(±)	+	−
Lactose[a]	±	±	+	(±)	+	−	+	−
Maltose	(±)	±	−	(±)	+	−	±	+
Melibiose[a]	±	±	−	+	(±)	(±)	±	−
Sucrose	+	(±)	−	(±)	(±)	−	+	+
Trehalose[a]	+	+	−	+	(±)	+	(±)	+
Raffinose[a]	±	±	−	±	(±)	−	(±)	−
Dextrin	−	(±)	−	±	−	−	−	−
Mannitol[a]	±	±	−	±	−	−	−	−
Amygdalin	±	±	−	±	−	NI	+	−
Aesculin	(±)	(±)	−	±	−	+	+	−
Salicin	(±)	(±)	−	−	−	(±)	+	−

[a] Tests suggested as the most useful in separating species.
± Variable reactions.
(±) Most strains positive, occasional strains negative.
(∓) Most strains negative, occasional strains positive.
NI, no information.

than one species of acidophilic leuconostoc. Peynaud and Domercq (1968) divided their isolates into two and Nonomura and Ohara (1967) into five species. However, despite phenotypic variation, it is probable that all acidophilic leuconostocs belong to a single genotype (Garvie and Farrow, 1980).

2. *Carbohydrate fermentations*

A basal medium for fermentation tests is given in Appendix I. It is based on that used by Garvie (1960). Sharpe *et al.* (1972) used a different basal medium which was designed for lactobacilli but is suitable for *L. mesenteroides*. The latter medium has an initial pH of 6.2 which is low for some leuconostocs, particularly *L. cremoris*. A pH of 6.7 is probably a better choice. Whittenbury (1963) used a semi-solid medium but this does not appear to be necessary. The additional information claimed from the use of such a medium is not essential to the interpretation of the fermentation patterns. The acid present in well grown cultures can change the indicator on inoculation into fermentation tests particularly when a heavy inoculation is required. Therefore, cultures should be centrifuged, the supernatant discarded and the cells taken up in sugar-free basal medium (or peptone water when the basal medium contains agar), equal to half the original volume. Up to 0.1 ml of this cell suspension can be inoculated into 5.0 ml of test medium without any change of indicator. This technique should also be used for *L. oenos*.

L. cremoris has a distinctive fermentation pattern as it can use only glucose, galactose and lactose. *Lb. viridescens* ferments fewer sugars than *Lb. confusus* so that these species resemble *L. dextranicum* and *L. mesenteroides*, respectively. *L. lactis* is adapted to live in milk and ferments lactose more readily than other species, in addition trehalose is generally not fermented. *L. paramesenteroides* has few differences from *L. mesenteroides* and was at one time considered to be a non-dextran forming variety of *L. mesenteroides*. It is easy to determine fermentation patterns but the variable results obtained with strains of the same species and the similarity of the patterns found with different species make it difficult to use fermentation patterns with confidence when identifying leuconostocs.

3. *Dextran production*

Neither *L. lactis* nor *L. paramesenteroides* form dextran, and this is a useful property in separating them from *L. mesenteroides*. Dextran production is observed on the surface of agar containing 5% sucrose. Cultures are incubated aerobically at 20–25°C for three to five days. The type of colony

formed depends on the chemical structure of the dextran and McCleskey *et al.* (1947) divided strains of *L. mesenteroides* into four groups on the basis of the type of colony formed on sucrose agar. Later workers have not used this property to divide strains, and since the type of dextran formed by different cells within a culture can vary (Brooker, 1977), the type of dextran formed is not taxonomically important. The dextran formed by *Lb. confusus* and *Lb. viridescens* has not been studied and nothing is known about the variation in the type of dextran formed by different strains.

4. *Lactic acid*

The type of lactic acid formed by lactic acid bacteria has long been used to separate the different genera and species. This property was not used widely in the first half of this century as the determination of lactic acid type was tedious; it was made quick and easy with the development of enzymic techniques. L(+)-Lactate is readily determined using mammalian lactate dehydrogenase (LDH). D(−)-Lactate can be determined by a similar technique and total lactic acid can be determined chemically. Thus DL-lactate production can be studied using two enzymic methods or using total acid together with L(+)-lactate. Leuconostocs only form D(−)-lactate from glucose, whereas heterofermentative lactobacilli form DL- and streptococci L(+)-lactate. Both L(+)-LDH and D(−)-LDH are available commercially and suppliers give details of conditions under which lactate can be assayed enzymically. An alkaline pH is necessary for the conversion of lactate to pyruvate but alkaline solutions will absorb CO_2 which can interfere with the assay. Storage of reagents in a desiccator with NaOH pellets should keep solutions CO_2 free.

DL forming bacteria may not produce equal amounts of both isomers (Garvie, 1967d). Only a small amount of lactate formed by *Lb. viridescens* consists of the L(+) isomer, but *Lb. confusus* forms almost equal amounts of D- and L-lactate. The media and conditions of growth can influence the proportion of each isomer and many bacteriological media contain measurable amounts of L(+)-lactate from peptone and meat extract. A high initial content of L(+)-lactate in media can be overcome if tomato juice, which is free of lactate, is used in place of meat products (Garvie, 1967d). The medium used in the study of the production of D- and L-lactate by lactic acid bacteria may appear deficient compared with MRS, but it supports good growth of most lactic acid bacteria including *L. oenos* for which species the pH is lowered to 5.0–4.8. When traces of one isomer of lactate are detected it is advisable to make a second determination at a higher concentration because there can be slight differences in the blank value of the reagent mix. In some techniques it is suggested that the reaction goes to completion and that it is

possible to calculate the amount of lactate from the increase in absorption at 340 nm. Experience with the technique has shown that it is better to include lactate standards (max 16 μg for the L isomer but 160 μg for the D isomer) in every series of determination, DL-lithium lactate can be used for both assays. All material examined has been found to contain equal amounts of each isomer. Most lactic acid bacteria reduce the pH of the medium to below 5.0 and Table IV gives an indication of the amount of lactate which will be formed when cultures are grown in dilute tomato broth (DTB, Table 1 in Appendix I). These values apply only to cultures with an initial pH of 6.7 and do not apply to *L. oenos*.

TABLE IV
Final pH, and lactate formed by leuconostocs growing in dilute tomato broth

Initial pH	Final pH	Amount of lactate (mg ml^{-1})	Dilution for testing[a]	
			L(+)-Lactate	D(−)-Lactate
6.7	5.0–4.7	2–4	1/50	1/5
	4.7–4.3	4–8	1/100	1/10
	4.3–3.9	8–16	1/200	1/20

[a] 0.2 ml of culture dilution in assay of 2.5 ml total volume.

L. oenos converts malate to lactate but it is not unique, and Alizade and Simon (1973) studied the fermentation of L- malate by *L. mesenteroides* ATCC 12291. They found that L-malate is converted to L(+)-lactate whereas glucose is converted to D(−)-lactate. The former reaction does not involve pyruvate and an LDH. It is essential, therefore, that the medium used for the determination of lactate from glucose is malate free. Other organic acids, for example citrate, which are metabolized to pyruvate can be included because pyruvate is only metabolized to lactate by an LDH.

C. Amino acid and vitamin requirements

All species of *Leuconostoc* require some preformed vitamins and amino acids. There are differences between the various species but they are not particularly useful taxonomically and any use is outweighed by the labour and expense of materials. For commercial operations, a knowledge of growth requirements can be very important both in fermentations used in making food products and in growing strains for converting sucrose to dextrans.

Garvie (1967c) described media and methods that were based on earlier studies, and gives results for several strains of every species. The two most important characters found were that *L. lactis* did not require folic acid whereas strains of other species showed a requirement, and *L. paramesenteroides* required riboflavin whereas *L. mesenteroides* did not. No purified TJF was available and tomato juice could not be incorporated in the medium. Many strains of *L. oenos* grew, and it is assumed that the conditions were correct and the pantothenate level high enough for it to be used in place of TJF.

D. Metabolism and enzyme studies

1. *General*

Modern recognition of bacterial species uses metabolic pathways and a study of key enzymes. These properties show little variation between strains of a species and are less variable than properties depending on the chemical ability of the growing cell. Obviously, the enzymes easiest to use are those of major importance to the life of the bacterium which have a readily available cheap substrate, and which can be directly assayed. Enzymes concerned with the few initial reactions, and the terminal reactions in the fermentation of glucose come into this category. A description of the pathway of glucose breakdown in leuconostocs is given in Section I. The exact method by which 6-phosphogluconate is decarboxylated and converted to ribulose-5-phosphate is uncertain (Yashima and Kitahara, 1969). Both glucose-6-phosphate dehydrogenase (G-6-PDH) and LDH can be directly assayed, are present in large amounts in cell free extracts, are stable and have relatively cheap substrates and coenzymes. The absence of an enzyme is more difficult to show than its presence but fructose-1,6-diphosphate aldolase can be used to separate heterofermentative from homofermentative lactic acid bacteria. G-6-PDH can also be used although some species of homofermentative lactobacilli possess this enzyme. LDH is present in all lactic acid bacteria and can be used to identify species of all the different genera.

2. *Lactate dehydrogenases*

Cells must be broken because the LDHs can only be assayed in cell-free extracts. Breakage can be achieved mechanically but some strains do not break readily. However, enough enzyme is usually obtained from cells of a 100 to 200-ml culture even when few cells appear disrupted. Different conditions of growth and breakage have been used by different workers but

these do not affect the enzyme (Sharpe *et al.*, 1972; Hontebeyrie and Gasser, 1975). Leuconostocs have a single LDH forming D(−)-lactate whereas heterofermentative lactobacilli have two LDHs and form D(−)- and L(+)-lactate. All D(−)-LDHs but only some L(+)-LDHs are reversible (Table V). At one time leuconostocs were classified as streptococci but the latter genus has FDP-dependent L(+)-LDHs, enzymes with many differences from the D(−)-LDHs of leuconostocs.

TABLE V
Development of lactate dehydrogenases of some lactic acid bacteria after electrophoresis

	L(+)-LDH			D(−)-LDH	
	Lactate	Pyruvate	Pyruvate + FDP	Lactate	Pyruvate
Streptococci	−	−	+	Absent	
Leuconostocs		Absent		+	+
Lactobacilli					
Lb. confusus, *Lb. viridescens*, *Lb. fermentum*	−	+	.	+	+
Lb. reuteri, *Lb. brevis*, *Lb. buchneri*	+	+	.	+	+

Enzymes can be recognized by differences in electrophoretic mobility and the technique developed for mammalian LDH was adapted for bacterial LDHs. The conditions of electrophoresis vary with different workers and details are given in the various publications (Garvie, 1969; Gasser, 1970). Where both L(+)- and D(−)-LDHs are present these can be identified using the appropriate lactate as substrate, providing the L(+)-LDH reacts with lactate. In *Lb. confusus* and *Lb. viridescens* the L(+)-LDH can only be located with pyruvate. The technique for locating the enzyme has been published (Garvie, 1969) and to achieve good results some precautions need to be taken. The amount of enzyme loaded should be carefully controlled as overloading results in a large unstained streak where the enzyme has travelled down the gel, with underloading the enzyme will be missed. These problems are less critical when using direct staining with lactate because the reaction can be stopped by immersing acrylamide gels in 5% acetic acid either after a few minutes (heavy loading) or after several hours development

(underloading). With pyruvate the reaction is invisible until the gels are transferred to the phenazine methosulphate/nitroblue tetrazolium solution. The initial stage has to be timed to get enough NADH into the gel for the purple colour reaction to show but not prolonged to get a blurred area of no colour. For acrylamide gels 30 min in the pyruvate/NADH solution is recommended.

The D(−)- and L(+)-LDHs are not separately identified using pyruvate and controls with NADH but no pyruvate should be included because NADH oxidases will react if they are present. In most strains NADH oxidases are weak and cause no reaction, but in the type strain of *L. mesenteroides* NCDO 523 and in strains of *Lb. viridescens* an active NADH oxidase has been detected (Sharpe *et al.*, 1972). In the latter species the NADH oxidase can mask the following weaker L(+)-LDH.

LDHs can be assayed by following NAD reduction at 340 nm. Most crude cell extracts are suitable for use with lactate and NAD. Leuconostoc LDH can be assayed with pyruvate providing allowance is made for NADH oxidase activity, but when two LDHs are present assays using pyruvate give little information. The various techniques used for assay and electrophoresis of bacterial LDH are given in a review by Garvie (1980).

This type of study has shown that the D(−)-LDH of the non-acidophilic leuconostocs *Lb. confusus* and *Lb. viridescens* has the same electrophoretic mobility, whereas *L. oenos* has a distinct LDH (Fig. 2). The L(+)-LDH is different in the two species of lactobacilli.

Lactic acid bacteria also have NAD-independent LDHs. These have been studied in several species (Garvie, 1980). The activity is weak and they are not useful in identifying *Leuconostoc* species.

3. *Glucose-6-phosphate dehydrogenase*

Glucose-6-phosphate dehydrogenase (G-6-PDH) can be assayed in the cell-free extracts prepared for LDH. The technique for handling the two enzymes is the same and, in many strains, both enzymes can be located in the same electrophoresis gel if both lactate and glucose-6-phosphate are included in the developing solution. In leuconostocs the G-6-PDH of non-acidophilic species can use either NAD or NADP as a coenzyme but with a preference for NAD. Electrophoresis does not separate the enzyme of the different species (Fig. 2). The G-6-PDH of *Lb. confusus* and *Lb. viridescens* can also use either NAD or NADP but on electrophoresis they are separated from the G-6-PDH of leuconostocs (Garvie, 1975). The G-6-PDH of *L. oenos*, on the other hand, uses NADP as a coenzyme, is difficult to detect after electrophoresis and, in some preparations, it has not been possible to demonstrate that it is present.

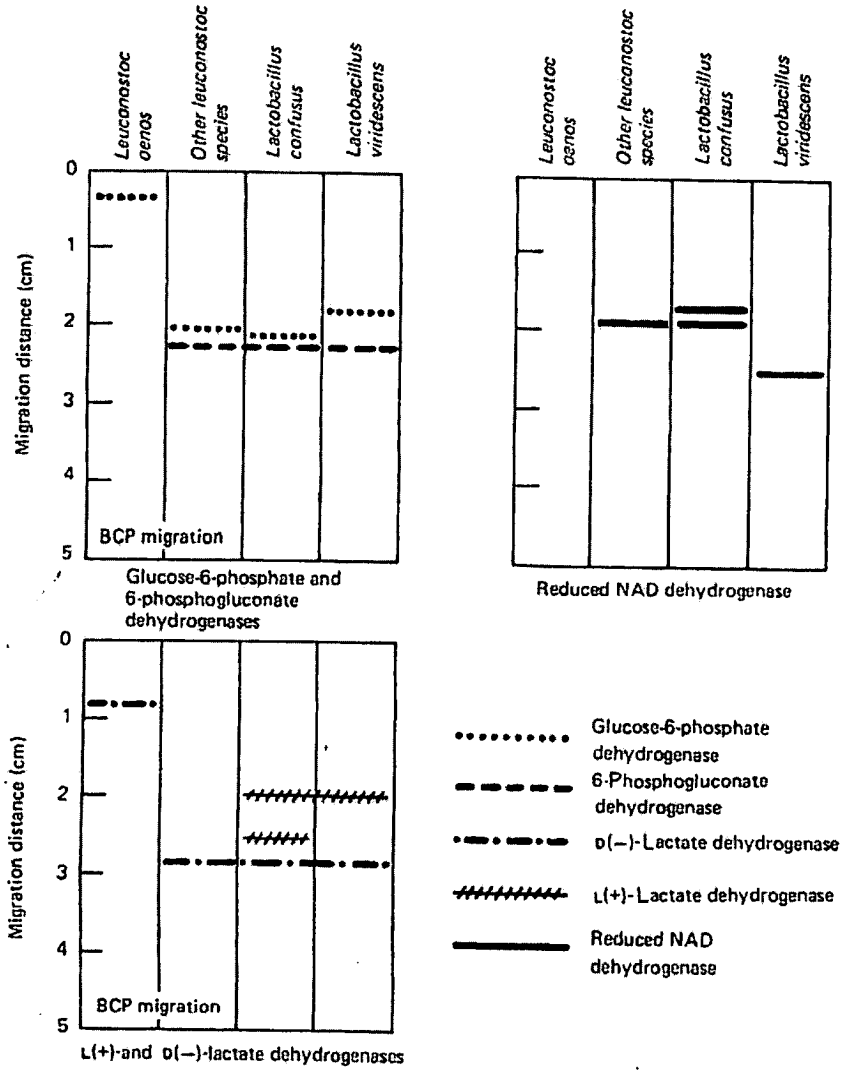

Fig. 2. The electrophoretic patterns of some dehydrogenases of the glycolytic system of leuconostocs and two species of lactobacilli.

4. *Immunological studies using dehydrogenases*

Hontebeyrie and Gasser (1973, 1975) have described methods of purifying both LDH and G-6-PDH and used the purified enzymes for inoculation into rabbits to prepare antisera active against the enzymes. New techniques using affinity chromatography have now been developed for dehydrogenases (Kelly *et al.*, 1978). Any future work should use these improved techniques.

Crude cell extracts can be tested against antisera and the techniques for this work have been described (Hontebeyrie and Gasser, 1975). Differences between the LDHs of the species of leuconostoc were found and also differences between the G-6-PDHs. The grouping of the strains examined was identical with both enzymes (Table VI). Seven groups were found: A contained *L. lactis*, F contained *L. paramesenteroides*, G contained *L. oenos*, D contained *L. dextranicum*, *L. cremoris* and most strains of *L. mesenteroides*. B, C and E were small groups each containing only one or two strains of *L. mesenteroides*. Many of the strains examined came from culture collections and more information, particularly using freshly isolated strains, is needed. This type of work is of great value in further separating enzymes which are electrophoretically identical. It is also useful as a basis for working out the evolutionary relationships between species.

TABLE VI

Immunological groups of D(−)-lactate dehydrogenase and glucose-6-phosphate dehydrogenases of leuconostocs

	Leuconostocs					
	L. mesenteroides					
	subsp. *mesenteroides*	subsp. *dextranicum*	subsp. *cremoris*	*L. lactis*	*L. paramesenteroides*	*L. oenos*
Lactate dehydrogenase	BCDE	D	D	A	F	G
Glucose-6-phosphate dehydrogenase	bcde	d	d	a	f	–

5. *Hybrid enzymes*

Bacteria seldom contain isoenzymes and in leuconostocs only a single band of each LDH is normally detected. Chilson *et al.* (1965) described a freeze-thaw technique for preparing hybrids using the tetrameric mammalian LDH. They showed the similarity in structure between the LDHs of several species. In leuconostocs the LDH of *L. oenos* and that of other species are well

separated by electrophoresis. Using the method described for mammalian L(+)-LDHs with the LDHs of leuconostocs, it was found that *L. oenos* LDH formed a single active hybrid enzyme with the LDH of any other species of the genus and also with the LDH of *Lb. confusus* and *Lb. viridescens*, but not with the LDH of other species of lactic acid bacteria. A weak hybrid was formed with the LDH of *L. lactis* and that of some leuconostocs (Garvie, 1975).

These observations show that the structure of the LDH of leuconostocs is a dimer. It further shows that the structure of the LDH of *L. oenos* is not too different from that of other species otherwise the hybrid protein would not be an active enzyme.

More evidence from extended studies might be useful. This technique does not require any enzyme purification but enzymes with the same or very similar electrophoretic mobility clearly cannot be used together unless one protein is modified.

E. Peptidoglycan types in cell walls

Most of the work on the peptidoglycan types in bacterial cell walls has been done in Munich by various workers (Schleifer and Kandler, 1972). A wide range of bacteria have been examined and so there is good evidence that information from the work is useful in bacterial taxonomy. All species of *Leuconostoc* and *Lb. confusus* and *Lb. viridescens* have a similar type of peptidoglycan which is different from that in other species of heterofermentative lactobacilli (Table VII). Reference to the techniques used are given by

TABLE VII

Peptidoglycan types of bacterial cell walls of leuconostocs and some heterofermentative lactobacilli

Species	Peptidoglycan
L. mesenteroides	L-Lys-L-Ser-L-Ala_2, L-Lys-L-Ala_2
L. dextranicum	L-Lys-L-Ser-L-Ala_2
L. cremoris	L-Lys-L-Ser-L-Ala_2
L. lactis	L-Lys-L-Ser-L-Ala_2, L-Lys-L-Ala_2
L. paramesenteroides	L-Lys-L-Ser-L-Ala_2, L-Lys-L-Ala_2
L. oenos	L-Lys-L-Ala-L-Ser, L-Lys-L-Ser-L-Ser
Lb. viridescens	L-Lys-L-Ala-L-Ser, L-Lys-L-Ser-L-Ala_2
Lb. confusus	L-Lys-L-Ala_2, L-Lys-L-Ala
Lb. fermentum	L-Orn-D-Asp
Lb. reuteri	L-Lys-D-Asp
Lb. brevis / *Lb. buchnerii*	L-Lys-D-Asp

Schleifer and Kandler (1972). Not all strains of leuconostocs within any species have the same murein type and there is considerable overlapping of type in different species so that the information about peptidoglycans is of greater value in showing which genera leuconostocs resemble rather than distinguishing species within the genus. *L. oenos* is not markedly different from other species which may appear to be in contrast to some of the information obtained from enzyme studies.

F. Nucleic acid studies

The study of nucleic acid in bacteria is widespread and is fundamental to any natural classification system. Indeed, the results from work with deoxyribonucleic acid (DNA) overrides conclusions from other information when there is a conflict of interpretations.

1. *%(G+C) content of DNA*

Good yields of high molecular weight DNA will only be obtained if cells are harvested at the correct time. This is usually the late logarithmic or early stationary phase of growth. Each strain used must be considered separately and the amount of inoculum, time of inoculum and conditions of incubation altered to suit the strain. The culture used for seeding the medium from which cells will eventually be harvested must also be considered. Different growth will be obtained if a mother culture in the early stationary phase is used as compared with one of the same optical density which has reached the stationary phase some hours before it is used. It is impossible to give precise conditions for every species for within a species different strains will require slightly different conditions.

L. mesenteroides, *L. dextranicum*, *L. lactis* and *L. paramesenteroides* do not grow rapidly and usually it is possible to obtain a suitable culture after overnight incubation (18 h) at 30°C using a 0.001% inoculum. *Lb. confusus* grows rapidly and may well be overgrown if the same conditions are used. Two techniques are possible, either a heavy inoculum followed by 3–5 h incubation or a small inoculum and the chilled inoculated media held for some hours in ice before incubation starts during the night. Slow growing species *L. cremoris* and *L. oenos* do not cause many problems. They can be left until there is reasonable opacity (between 24 and 48 h for *L. cremoris* but seven days has been used for strains of *L. oenos*). DNA extracted from *Lb. viridescens* usually has a low hypochromicity (about 0.30) and yields are low.

Leuconostocs are sensitive to lysozyme providing the enzyme has time to act. Lysozyme binds to cells washed free of salt (Metcalf and Deibel, 1973) and this is recommended, although it can make pelleting of cells on

centrifuging difficult. Sometimes it is necessary to work from an initial cell slurry rather than a pellet. When Marmur (1961) developed a technique for preparing bacterial DNA he chose to work at pH 8.0 with EDTA in order to inhibit DNAase. Lysozyme has little activity at pH 8.0 and lactic acid bacteria do not have extracellular DNAase. When cells are difficult to lyse, it is better to work at pH 7.0 and it was found that 4-amino salicylate is a better additive than EDTA (Garvie, 1976). Other workers have used different conditions (Hontebeyrie and Gasser, 1977). Lysozyme is not attacked by proteolytic enzymes and, therefore, it is possible to add pronase with lysozyme and allow both enzymes to work overnight at 37°C.

Many procedures have been described for purifying DNA from contaminating protein and RNA and strains vary in the ease with which this can be done. A highly purified, high molecular weight DNA is essential for DNA/DNA hybridization work (DeLey and Tutgat, 1970). The % (G+C) content of DNA can be measured by melting temperatures even if some impurities are present, and purification is not essential when the %(G+C) content of DNA is estimated from buoyant density. Few publications give figures for protein and RNA contamination but these should be measured chemically until the technique chosen has been shown to be adequate. Generally, there should be less than 5% (w/w) protein or RNA in DNA. Some techniques involve precipitating DNA with ethanol and, if a method of this type is selected, problems may arise in dispersing the DNA for the next stage of purification. The method recommended by Kirby *et al.* (1967) is probably the most satisfactory, that is centrifuging a viscous solution adding more solute to the precipitated DNA and repeating the procedure. Complete dispersal of DNA before estimating the melting temperature is also essential. Solutions suitable for storage have an optical density at 260 nm of at least 20 and so considerable dilution is necessary. Freshly prepared DNA is sometimes difficult to disperse, whereas preparations stored for two to three months present no problems. Holding the diluted DNA at 37°C for 60 min with occasional gentle shaking will probably result in complete solution.

Escherichia coli K12 is suitable as a control DNA, but *L. mesenteroides* NCDO 768 (ATCC 12291) is probably better. The standard formula for calculating %(G+C) content of DNA from T_m is based largely on results obtained by Marmur and DeLey (DeLey, 1970). Both these laboratories found the T_m for *E. coli* was 90.6°C. Marmur also used *L. mesenteroides* ATCC 12291 and obtained a figure of 86.5°C. Other laboratories obtain figures 1°C higher so that it is necessary to use a control DNA and to adjust the calculation according to the figures obtained for the control. The reason for the discrepancy is not known.

The %(G+C) content of DNA from different species is shown in Table VIII.

TABLE VIII

The % (G+C) content of the deoxyribonucleic acid of species of the genus *Leuconostoc* and of two species of lactobacilli expressed as a percentage

Species	% (G+C)
L. mesenteroides	38–40
L. dextranicum	38–39
L. cremoris	38–40
L. lactis	42–43
L. paramesenteroides	38–39
L. oenos	39–41
Lb. viridescens	41–42
Lb. confusus	44–45

2. *DNA/DNA hybridization*

Two studies have been made of the genus *Leuconostoc*. Garvie (1976) used the membrane filter method of Denhardt (1966), whereas Hontebeyrie and Gasser (1977) used an hydroxyapatite method. The results of the two studies are in agreement. The choice of technique is up to the laboratory since both methods have been well tested with DNA from many species. The results obtained from this work are summarized in Table IX and the implications are discussed in Section V.

3. *RNA/DNA hybridization*

Ribonucleic acid (RNA) evolves slowly and studies using RNA are valuable in showing the relationships between species and genera which are separated by DNA/DNA hybridization (Fox *et al.*, 1977). A number of genera of Gram-negative bacteria have been examined (Gillis and DeLey, 1980) but the Gram-positive bacteria, particularly the lactic acid bacteria, have not been examined. Ribosomal ribonucleic acid (rRNA) is present in large amounts in bacteria and can be extracted from lysed cells by the technique of Moore and McCarthy (1967); Garvie and Farrow (1981) have described a technique for lysing cells of Gram-positive cocci before extracting RNA. The techniques of studying RNA/DNA hybridization are still being developed. Present methods are described by DeLey and DeSmedt (1975) and by Gillespie (1968). Studies with lactic acid bacteria are required. Some preliminary work with leuconostocs has indicated that the RNA of all the non-acidophilic strains is highly related but different from that of *L. oenos* and also that of *Lb. confusus* and *Lb. viridescens*. There is only a low relationship between the two lactobacilli as judged by rRNA/DNA hybridization despite the fact that they

TABLE IX

Summary of hybridization between the deoxyribonucleic acids of strains of leuconostoc and lactobacilli

Species NCOD Number	Hybridization (%) of labelled DNA from				
	L. mesenteroides 523	*L. mesenteroides* 768	*L. lactis* 546	*L. paramesenteroides* 803	*Lb. viridescens* 403	*Lb. confusus* 1586
L. mesenteroides A	80–100	30–40	20–40	8–18	15–25	13–17
B	30–40	90–100	25–35	14–20	15	15
L. dextranicum	80–100	30–45	20–35	5–20	15–35	10–20
L. cremoris	70–100	45	25–35	7–8	12–21	13
L. lactis	30–50	28–60	74–100	—	20–50	18–30
L. paramesenteroides	6–20	13–21	10–15	60–100	15–20	27
L. oenos	10–15	10	0–10	5	1	1
L. viridescens	12–25	—	18–20	—	90–100	30–50
Lb. confusus	15–20	—	10–20	—	30–65	80–100

are both heterofermentative lactobacilli (Garvie, 1981).

Messenger RNA (mRNA) is not suitable for hybridization studies. It is not possible to obtain it free from large amounts of rRNA. Yields of mRNA are low and reflect only the RNA involved in the requirements of the bacteria at the time of labelling, usually in the mid logarithmic phase of growth.

G. Serology and bacteriophage typing

Separation of species and strains of non-pathogenic bacteria by serotyping is not a satisfactory technique. In the lactic acid bacteria its use is restricted to species of pathogenic streptococci and some other species also associated with animals.

Bacteriophage typing of host bacteria is restricted to those species which are readily attacked by bacteriophage. Slow growing bacteria seldom occurring in large populations are not susceptible to bacteriophage attack. Leuconostocs belong to this type of bacteria and consequently while leuconostocs have long been found in association with streptococci in cheese starter culture they are not, normally, attacked by bacteriophage. A single report (Sozzi *et al.*, 1978) described leuconostoc bacteriophage.

Leuconostocs are now cultured commercially for the production of dextran, but the dextran itself is believed to protect the bacteria from bacteriophage attack. If this is true, trouble in industry is unlikely.

Bacteriophage attacking wild populations, if they do exist, will be present in small numbers and difficult to isolate. Leuconostoc bacteriophage are, therefore, unavailable for typing strains.

IV. Taxonomy

Bacterial classification is primarily based on morphology, therefore the lactic acid bacteria fall into two families: the cocci are *Streptococcaceae* and the rods are *Lactobacillaceae*. For practical purposes this separation is not helpful. The homofermentative cocci dividing in one plane, the streptococci, occupy a different ecological niche from the cocci dividing in two planes, the pediococci, and from the heterofermentative cocci, the leuconostocs. These last two genera can be found in the same habitat as the lactobacilli (both heterofermentative and homofermentative) and in many ways a family comprising these three genera might be preferable to the present divisions.

The heterofermentative and homofermentative cocci have been accepted as belonging to different genera from the time Hucker and Pederson (1930) studied leuconostocs. The same division has not occurred in the rods—possibly because the latter occur together and have always been studied as a

single group. The separation of homo- and heterofermentative organisms into different genera makes evolutionary sense since the essential pathway of sugar metabolism is different in the two groups suggesting that the organisms have been separated for a long time.

The relationship of leuconostocs to the heterofermentative lactobacilli still awaits clarification but the difficulty of separating *Lb. confusus* and *Lb. viridescens* from leuconostocs indicates that they may all belong to a single genus. The other species of heterofermentative lactobacilli have a different cell wall peptidoglycan, different LDHs and G-6-PDHs from those of *Lb. confusus* and *Lb. viridescens*, so there are arguments against forming a single genus to include all the heterofermentative lactic acid bacteria.

The first clear separation of leuconostocs into species resulted from the work of Hucker and Pederson (1930) who showed that *L. mesenteroides* fermented pentoses and sucrose. *L. dextranicum* fermented sucrose but not pentose and *L. cremoris* (*citrovorum* in 1930) did not ferment either pentose or sucrose. This separation was not wholly satisfactory and the reason is now evident because all these species have high homology using DNA/DNA hybridization and form a single geno-species. *L. lactis* and *L. paramesenteroides* were recognized later (Garvie, 1960, 1967c) but may well have been isolated long before they were recognized as separate species, and classified as either *L. mesenteroides* or *L. dextranicum*.

Confusion with named species also occurred with *L. oenos*, and isolations from wine prior to 1967 used the names of non-acidophilic species, probably incorrectly. Lactobacilli found in wine are also found in other habitats and there was no reason to think that the leuconostocs found in wine and non-acid environments were different species. Leuconostocs of wine have so far not been isolated from other environments.

Classification of bacteria from natural habitats cannot rely on elaborate techniques. In leuconostocs it is difficult to select a few tests which will be satisfactory and an attempt has been made to classify the genus using multivariate analysis. When information of the type normally used in numerical taxonomic studies was analysed a clear separation into groups was not achieved, the majority of strains of *L. mesenteroides*, *L. dextranicum*, *L. paramesenteroides* and *L. confusus* came in a single cluster. *L. lactis* and *Lb. viridescens* were in separate clusters, whereas *L. cremoris* and *L. oenos* were separated from other species but not from each other. In Table III the tests considered of most use in separating the different species are marked by a footnote.

A possible future grouping of the heterofermentative lactic acid bacteria is given below, by priority the name of the genus would be *Leuconostoc* leaving *Lactobacillus* for the homofermentative rods.

Subgenus 1. *Leuconostoc oenos*

Subgenus 2. *Leuconostoc mesenteroides* subsp. *mesenteroides*, subsp. *dextranicum*, subsp. *cremoris* (Garvie, 1983)
Leuconostoc paramesenteroides
Leuconostoc lactis

Subgenus 3. *Lactobacillus confusus*
Lactobacillus viridescens

Subgenus 4. *Lactobacillus fermentum*
Lactobacillus reuteri
Lactobacillus brevis
Lactobacillus buchnerii

Classification is a developing science and as knowledge increases it may well change to accommodate the new facts.

V. Commercial importance

A. Introduction

The commercial importance of leuconostocs can be divided into two categories: natural and manufactured. In the first category (natural) the bacteria grow and the changes resulting from this growth are important; in the sugar industry spoilage occurs but in the dairy and wine industries the fermentations are beneficial. It is necessary to be able to identify the bacteria responsible; in some instances, particularly in the sugar and wine industries, the bacteria are natural contaminants, whereas in the dairy industry leuconostocs are kept as cultures, but can also occur naturally in milk and cheese. The second category (manufactured) concerns the production of dextran by *L. mesenteroides*. In this work it is necessary to be able to identify and keep stable the strain(s) used, and to ensure that they are free of contamination from wild populations.

The different species of leuconostoc are each adapted to a particular habitat and there is little overlapping, except in the dairy industry and on herbage. A knowledge of the growth characteristics of the various species will help to eliminate the sort of mistakes in identification that have occurred in the past.

B. Sugar industry

L. mesenteroides grows actively on sucrose and forms large amounts of dextran, whereas *L. dextranicum* is a less active organism and forms only

small amounts of dextran. Both species can cause spoilage in sugar cane and sugar beet after harvest. The sucrose content of the crop is reduced and the dextran interferes with extraction of sucrose (Sidebotham, 1974). The bacteria are active at atmospheric temperature particularly that of the cane fields. *Lb. confusus* also forms dextran actively from sucrose and has been isolated from sugar cane (Sharpe *et al.*, 1972). The problem of spoilage has been largely overcome by matching harvesting to processing.

Dextran production by *L. mesenteroides* can be beneficial and was reported to control ergot when the bacterium was growing in the honey dew of rye (Mantle, 1965). However, this appears to be an isolated occurrence.

C. Dextran formation

Dextrans (*D. glucans*) have many commercial uses and have been extensively studied as can be judged from the bibliography compiled by Jeanes (1978). She lists 2455 papers with an additional 938 dealing with patents. Most of the workers have studied *L. mesenteroides* dextrans, all of which are formed by a D-glucose chain with substantial (1→6) linked α-D-glucopyranosyl residues, but branching of the side chain occurs (Sidebotham, 1974). Different strains form different dextrans and within a population cells may not all behave alike (Brooker, 1977). Much of the early work classifying the types of dextrans formed by *L. mesenteroides* was done in the Northern Regional Research Laboratories at Peoria (Jeanes *et al.*, 1954) and the culture collection at Peoria holds a wide variety of strains.

Dextrans are proving to be useful in research, industry and medicine. Modified dextrans have become widely used as gels used in filtration work to separate compounds of different molecular weights. They are used for purification and for determining molecular weights. The former application covers desalting and removal of small molecular weight material from larger molecules and can be used on any scale from a few millilitres to an industrial process. Derivatives of dextrans have been developed for a multiplicity of special purposes. In the medical field these include use as adjuvants, to aid interferon induction and to increase infectivity of viruses. Dextrans can be used in perfusion studies and also as carriers of pharmacologically active substances as insulin and vitamin B_{12} and for enzyme stabilization.

From this brief indication of the uses of dextran it is clear that *L. mesenteroides* has become an essential tool in many modern processes and without the ability of the bacteria to convert sucrose to dextran progress in biochemistry and medicine would probably have been slower.

D. Dairy industry

Leuconostocs occur with streptococci in milk and dairy products and in this field the two genera have not always been separated as morphologically they are indistinguishable. Lactose-fermenting species, *L. lactis* and *L. cremoris*, are the most important but other non-acidophilic species are sometimes found as minor components of milk and other dairy products. In the early part of this century leuconostocs were classified as streptococci (Hammer, 1920) but at about the same time Orla-Jensen (1919) recognized both betacocci (leuconostocs) and streptococci.

Both *L. lactis* and *L. cremoris* are included in cheese and butter starters although many starters consist entirely of streptococci. Leuconostocs are slow growing and not important in the conversion of lactose to lactic acid, but in forming flavour components in the acid ferment (Drinan *et al.*, 1976). Leuconostocs grow in milk only in association with streptococci and the flavour components are found after the pH has been lowered by the streptococci. These flavour components result partly from the heterofermentation of carbohydrate but mostly from the breakdown of citrate. Leuconostocs which cannot use citrate have no value in starters. All strains of *L. cremoris* examined can use citrate, and it is of interest to note that this species has only been isolated from dairy products. *L. mesenteroides* uses lactose very slowly and *L. cremoris*, now believed to be a variety of *L. mesenteroides*, appears to have adapted to live in milk. The ability of leuconostocs to use citrate is important to the dairy industry and few of the strains examined (Garvie, 1967a) used citrate, whereas earlier reports (Hucker and Pederson, 1930) suggested that many strains of *L. mesenteroides* were able to do so. The difference in these findings is striking but can be explained by the use of recently isolated strains in the older work as against old laboratory strains in the more recent work. Clearly, information about the stability of the ability to use citrate is important to the dairy industry.

E. Wine industry

When grape juice is made into wine the malic acid in the juice is converted to L(+)-lactate (malo-lactic fermentation) with a consequent rise in pH. This fermentation is essential in making wine, and a variety of bacteria have the necessary enzymes including both *L. oenos* and *L. mesenteroides*. When the malo-lactic fermentation was first recognized as important it was not realized that leuconostocs were a species peculiar to wine (Kunkee, 1967). *L. mesenteroides* is not important in wine fermentation as it will not grow at the low pH of grape juice nor in the ethanol formed by yeast. Peynaud and

Domercq (1968) report that *L. oenos* is the most important organism involved in malo-lactic fermentation under vinification conditions.

L. oenos is clearly important to the wine industry but much of the fermentation is left to the flora which develops naturally. *L. oenos* is a species which contains strains with different fermentation patterns, different vitamin and amino acid requirements and different growth rates in laboratory culture. All these factors could influence the growth of naturally occurring bacteria, and affect the quality of the wine. The separation of *L. oenos* into different subspecies (or possibly different species) is important if the production of wine is to be understood. More work, particularly on the metabolic pathways, the enzyme proteins and nucleic acids of *L. oenos* is needed.

To what extent the end-products (apart from lactate) formed by *L. oenos* also influence wine is not clear but if *L. cremoris* is a flavour producer in butter *L. oenos* may also be a flavour producer in wine.

Appendix I
Media used in the identification of species of the genus *Leuconostoc*

1. *Percent (w/v) of ingredient of general media*

Ingredient	Medium identification code						
	YGPB	MRS	Medium 1	Medium 2	ATB	CMB	DTB
Glucose	1.0	2.0	1.0	1.0	1.0	1.0	1.0
Peptone	1.0	1.0	1.0	1.0	1.0	1.0	0.75
Meat extract	0.8	0.8	—	—	—	—	—
Yeast extract	0.3	0.5	0.5	0.5	0.5	0.5	0.25
NaCl	0.5	—	—	—	—	—	—
KH_2PO_4	0.25	—	0.5	0.5	—	0.25	0.25
K_2HPO_4	0.25	0.2	—	—	—	—	—
$MgSO_4.7H_2O$	0.2	0.2	0.2	0.2	0.2	0.2	0.2
$MnSO_4.4H_2O$	0.005	0.005	0.005	0.005	0.005	0.005	0.005
Ammonium citrate	—	0.2	0.5	—	—	—	0.1
Citric acid	—	—	—	0.5	—	0.25	—
DL—Malic acid	—	—	—	—	—	0.25	—
Sodium acetate	—	0.5	0.25	0.25	—	—	0.25
Tween 80	—	0.1	0.1	0.1	—	0.1	0.1
Tomato juice	—	—	—	10.0	25.0	—	10.0
Usual pH	6.8	6.2	6.5	4.8	4.8	4.8	6.5

continued on p. 175

1.5% agar is added when solid media are required.
0.05% cysteine hydrochloride is added to all media when required.
Sterilization is normally at 15 lb for 15 min.
Special techniques used with different media are mentioned in the text.
YGPB: yeast glucose phosphate broth (Garvie, 1976).
ATB: acid tomato broth (Garvie and Mabbitt, 1967).
CMB: citrate-malate broth (Garvie and Mabbitt, 1967).
DTB: dilute tomato broth (Garvie, 1967d).
MRS: (DeMan *et al.*, 1960).
Mediums 1 and 2: (Garvie, 1969).

2. *Milk agar for testing for gas production*

a. Litmus milk	800 ml	
Glucose	5.5 g	
Oxoid yeast extract	2 g	
b. Oxoid tomato juice	100 ml	
c. Nutrient agar	200 ml	(Oxoid Nutrient Broth No. 2 with 1.5% agar)

To prepare, mix a and b, adjust to pH 6.8, warm to 45°C and add c melted. Dispense in 10-ml amounts and autoclave 10 lb for 10 min. Water cool. For use, melt, cool to 45°C and inoculate with 0.25 ml of culture, solidify in cold water and layer on 4.0 ml of nutrient agar.

3. *Sucrose agar for dextran production*

Oxoid tryptone	1.0%
Yeast extract	0.5%
K_2HPO_4	0.5%
Di-ammonium citrate	0.5%
Sucrose	5.0%
Agar	1.5%
pH 7.0	Autoclave 15 lb for 15 min

4. *"Sugar" basal broth*

Oxoid peptone	1.0%
Yeast extract	0.25%
Tween 80	0.01%
Bromocresol purple (1.6% sol in ethanol)	0.1%

pH 6.8. Tube in 5.0-ml amounts and autoclave 15 lb for 15 min
See p. 154 for modification for use with *L. oenos*

Carbohydrate substances are prepared as 2% (w/v) solutions (1.0% for aesculin and inulin). Autoclave 10 lb for 10 min and add 0.5 ml to 5.0 ml of basal medium.

5. *Acetate agar*

BBL Trypticase	1.0%
Arabinose	0.5%
Glucose	1.0%
Sucrose	0.5%
Yeast extract	0.5%

KH_2PO_4		0.6%	
Di-ammonium hydrogen citrate		0.2%	
Tween 80		0.1% (v/v)	
Salt solution 1		0.25% (v/v)	
Salt solution 2		0.25% (v/v)	
Agar		1.5%	
Salt solution 1.	$MgSO_4.7H_2O$	57.5 g	in 250 ml of H_2O
	$MnSO_4.4H_2O$	12.0 g	
Salt solution 2.	$FeSO_4.7H_2O$	3.4 g	in 250 ml of H_2O
	HCl (conc)	5.0 ml	
Buffer solution pH 5.4	Sodium acetate $3H_2O$	25 g	+ 160 ml of H_2O
	10% glacial acetic acid	40 ml	
	Use at 200 ml l^{-1} litre medium		

To make

A Dissolve agar in water (15 g to 500 ml of H_2O) by steaming.
B Dissolve other ingredients for 1 litre medium in 300 ml of H_2O.
C Mix these two solutions and steam for 10 min (= 800 ml of H_2O).
Add 200 ml of buffer solution to C while hot.
Dispense in sterile containers. Do not autoclave.

6. *Whey serum agar with calcium citrate*

i. Whey is made with rennet from milk.
ii. Serum is obtained from filtering a cheese starter culture.
iii. Whey and serum are mixed (1:1) neutralized to pH 7.3 with $Ca(OH)_2$ suspension. Steamed for 30 min, filtered and the pH readjusted to 7.3 with NaOH. $MnSO_4$ (probably 0.005% w/v. the paper is unclear) and 1.5% agar are added, the medium cleared with albumin and sterilized (15 min 10 lb).
vi. Calcium citrate suspension—1.5% carboxymethylcellulose (viscosity 60–120 cp at 20°C 1% solution) is dissolved in water at 45–50°C. 10 g of finely powdered calcium citrate are suspended in 100 ml of carboxymethylcellulose solution. This suspension is held at 45°C for 1.5–2 h. The coarse particles which have sedimented are discarded. The fine suspension is sterilized (10 min 15 lb). (The concentration of calcium citrate can be checked by OD at 750 nm. 1/100 dilution should be 0.8–0.9 using a 1-cm light path.) 1 ml of calcium citrate suspension is added to 15 ml of whey serum agar for use. (If the calcium citrate has precipitated during storage it should be gently mixed before use.)

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6

Fatty Acid and Carbohydrate Cell Composition in Pediococci and Aerococci, and Identification of Related Species

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I. Introduction

Gram-positive cocci constitute a significant part of environmental contaminants, for instance in pharmaceutical production, brewing and wine making. Aerococci and pediococci have, for instance, been isolated from medicine bottles (Clausen, 1964), but may be present in a number of plant raw materials used in production and aerococci constitute an important part of air contaminants. These organisms are found in brewers yeast, beer, wine and fermenting mashes such as sauerkraut, pickles and silage. These

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5 The genus *Pediococcus*, with notes on the genera *Tetratogenococcus* and *Aerococcus*

W.J. SIMPSON and H. TAGUCHI

5.1 Introduction

Pediococci are the only lactic acid bacteria that divide alternately in two perpendicular directions to form tetrads (Figure 5.1). They are invariably spherical and produce lactic acid, but no gas, from glucose. The genus is heterogeneous and includes organisms able to grow in beer and those active during soya sauce manufacture. A number of papers address various aspects of the genus (Pederson, 1949; Pederson *et al.*, 1954; Nakagawa and Kitahara, 1959; Sakaguchi and Mori, 1969; Garvie, 1974, 1986a; Eschenbecher and Back, 1976; Back, 1978a; Rainbow, 1981; Bergan *et al.*, 1984; Priest, 1987; Raccach, 1987; Weiss, 1991; Teuber, 1993) which currently contains eight species. Information on the genus *Aerococcus* can be found in the review by Weiss (1991). Table 5.1 lists the species of pediococci, together with common synonyms and a brief description of

Figure 5.1 Tetrad formation of pediococci. The scanning electron micrograph shows cells of *Pediococcus pentosaceus* BSO 347 (BSO, beer-spoilage organism collection, BRF International, UK). Scale bar represents 1 µm.

Table 5.1 Names, synonyms and descriptions of *Pediococcus* species

Species and priority	Type strain*	Synonyms	Description
Pediococcus acidilactici (Lindner, 1887)	DSM 20284 (proposed by Garvie (1986b))	'*Pediococcus linderi*' '*Pediococcus cerevisiae*' '*Streptococcus lindneri*'	Grow at 50°C. Ferment ribose, arabinose and/or xylose. Unable to utilize maltose; DL-lactate produced from glucose. Hydrolyse arginine. Mol% G+C 38–44. Associated mainly with plant materials.
Pediococcus damnosus (Claussen, 1903)	NCDO 1832† (ATCC 29358; DSM 20331)	'*Pediococcus cerevisiae*' '*Pediococcus cerevisiae* subsp. *mevalovorus*' '*Pediococcus viscosus*' '*Pediococcus perniciousus*' '*Pediococcus sarcinaeformis*' '*Pediococcus odoris mellisimilis*' '*Pediococcus mevalovorus*' '*Streptococcus damnosus*' '*Streptococcus damnosus* var. *limosus*'	Unable to ferment ribose or hydrolyse arginine. No acid from starch, no acid or gas from gluconate, no growth at pH 8.0 or at 35°C. Most strains hop-tolerant and able to grow in beer; DL-lactate produced from glucose. Mol% G+C 37–42. Associated mainly with beer and breweries.
Pediococcus dextrinicus (Coster and White, 1964; Back, 1978b)	DSM 20335 (NCDO 1561; ATCC 33087)	'*Pediococcus cerevisiae* subsp. *dextrinicus*' '*Streptococcus damnosus* var. *diastaticus*'	Unable to ferment ribose or hydrolyse arginine. Acid from starch, acid and gas from gluconate, growth at pH 8.0. L(+)-Lactate produced from glucose. Mol% G+C 40–41. Associated with fermenting plant materials.
Pediococcus halophilus (Mees, 1934)	NCDO 1635 (ATCC 33315; DSM 20339)	'*Pediococcus soyae*' '*Pediococcus acidilactici* var. *soyae*' '*Tetracoccus no. 1*' '*Tetracoccus halophilus*' '*Sarcina hamaguchiae*' '*Tetratogenococcus halophilus*'	Grow in presence of 15% NaCl and at pH 9.0. L(+)-Lactate produced from glucose. Mol% G+C 34–36.5. Associated with salty environments.
Pediococcus inopinatus (Back, 1978a)	DSM 20285	'*Pediococcus cerevisiae*'	Unable to ferment pentoses and lactose. Does not hydrolyse arginine. DL-Lactate produced from glucose. Mol% G+C 39–40. Associated with beer and alcoholic beverages.
Pediococcus parvulus (Günther and White, 1961a)	NCDO 1634 (ATCC 19371; DSM 20332)	None	Grows at pH 4.5. Unable to utilize pentoses, lactose or starch. Does not hydrolyse arginine. Forms DL-lactate from glucose. Mol% G+C 40.5–41.6. Associated with fermented plant materials, cider and wine.
Pediococcus pentosaceus (Mees, 1934)	NCDO 990 (ATCC 33161; DSM 20336)	'*Pediococcus hennebergi*' '*Pediococcus citrovorum*' '*Pediococcus cerevisiae*' '*Pediococcus acidilacti*' '*Streptococcus acidi-lactici*'	Ferment pentoses (except strains belonging to *P. pentosaceus* subsp. *intermedius*). Ferment maltose. Do not ferment starch or melizitose. Hydrolyse arginine. Maximum temperatures for growth 39–45°C. DL-Lactate produced from glucose. Mol% G+C 35–39. Associated with plant materials.
Pediococcus urinae-equi (ex Mees) nom. rev.	NCDO 1636 (ATCC 29723; DSM 20341)	'*Pediococcus cerevisiae* var. *urinae-equi*' '*Pediococcus urinae-equi*' '*Aerococcus viridans*'	Grow at pH 9.0. Produces L(+)-lactate from glucose. Grow in the absence of fermentable carbohydrate. Mol% G+C 39.6–39.7. Associated with horse urine and animal faeces.

*DSM; Deutsche Sammlung von Mikrorganismen, Munich, Germany. NCDO; National Collection of Dairy Organisms, Reading, UK. ATCC; American Type Culture Collection, Rockville, Maryland, USA.
†Type strain of the genus.

each. One of these (*P. urinae-equi*) clearly belongs to the genus *Aerococcus* and proposals have been made that *P. halophilus* should also be placed in a new genus, '*Tetratogenococcus*' (Collins *et al.*, 1990). If the suggestions to exclude these acid-sensitive species from the genus are adopted, then the general description might be altered to read 'pediococci are the only acidophilic, homofermentative, lactic acid bacteria that divide alternately in two perpendicular directions to form tetrads'.

5.2 Morphology

In a single culture, the cells of pediococci are spherical and of uniform size, 0.36–1.43 μm in diameter (Günther and White, 1961a). They are never elongated. In contrast, cells of *Leuconostoc* spp. are often elongated and arranged in chains.

The mode of division of pediococci has frequently been the subject of dispute. Balcke (1884) stated that the cells divided in *one plane* and, in recognition of this, derived the name *Pediococcus* from two Greek nouns: *pedium*, meaning a plane surface, and *coccus*, meaning a berry. Later descriptions of tetrad-forming cocci described them as dividing in two planes, rather than in one, as Balcke had suggested (Günther, 1959; Herrmann, 1965). This may have been to differentiate their mode of division from that of chain-forming streptococci. Others, notably Shimwell (1948a, 1949), disputed the fact that tetrad formation in pediococci was the result of an unusual method of cell division, proposing that the cells divided normally to form chains, but then re-arranged to form tetrads. Shimwell (1949) pointed out that tetrads were more noticeable than chains of cells, and this may have explained the preoccupation of bacteriologists with such morphological features. Günther (1959) used time-lapse photography to show that tetrad formation did not result from rearrangement of the cells. However, she interpreted the mode of division as being in two planes. Pediococci are spherical, so it is not possible for them to divide in more than one plane while undergoing only two cell divisions (Simpson, 1994) since a plane is defined *as a surface containing all straight lines passing through a fixed point and also intersecting a straight line in space*. Thus, at any point in each of the two divisions needed to form a tetrad, the centres of all cells must lie within one plane (Figure 5.2). Of course, the cells must divide in two *directions*, each approximately at right angles to the other.

Pediococci are nonmotile, do not form spores and are not capsulated. When grown on a rich medium, such as de Man, Rogosa, Sharpe (MRS) agar (de Man *et al.*, 1960), the colonies are typically 1–3 mm in diameter, generally smooth-edged and invariably not pigmented (Back, 1978a). In stab culture, the cells grow along the line of the stab with little surface growth.

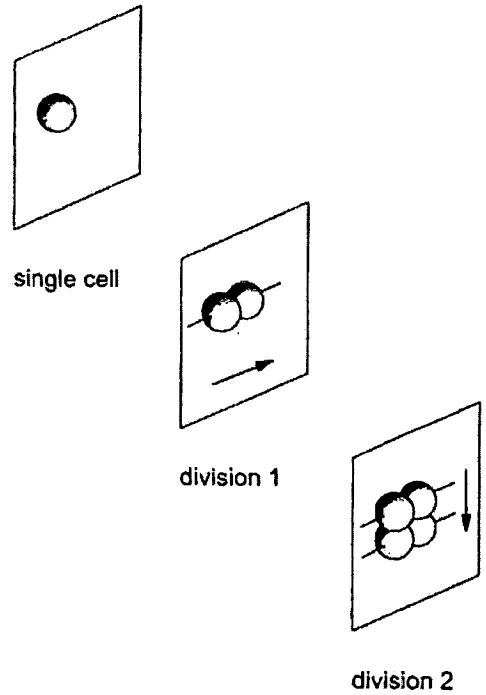


Figure 5.2 Tetrad formation takes place by division of the cells in two directions in a single plane.

In broth culture, growth is uniform throughout the medium (Nakagawa and Kitahara, 1959).

5.3 Physiology

5.3.1 Carbohydrate metabolism

Under anaerobic conditions, pediococci ferment glucose to give optically inactive (DL) or dextrorotary (L[+]) lactate. A wide range of carbohydrates can be used by various species, ranging from pentoses such as arabinose, ribose and xylose; hexoses, such as fructose and mannose; disaccharides, such as maltose; trisaccharides, such as maltotriose; and polymers, such as starch (Table 5.2). All species, except *P. urinae-equi*, are unable to grow in the absence of carbohydrate (Deibel and Niven, 1960). *Pediococcus pentosaceus* transports glucose using the phosphoenolpyruvate:phosphotransferase system and metabolizes it via the Embden–Meyerhof–Parnas pathway (Romano *et al.*, 1979). The metabolism of glucose by other pediococci has not been reported. Under certain conditions, metabolic

Table 5.2 Physiological characteristics of the pediococci*

Character†	*P. acidilactici*	*P. damnosus*	*P. dextrinicus*	*P. halophilus* (syn. '*Tetragenococcus halophilus*')	*P. inopinatus*	*P. parvulus*	*P. pentosaceus*	'*P. pentosaceus* subsp. *intermedius*'	*P. urinaeequi*
Growth at 35°C	+	−	+	+	+	+	+	+	+
40°C	+	−	+	+/− (weak)	+/− (weak)	−	+/− (weak)	+/− (weak)	+
45°C	+	−	+/− (weak)	−	−	−	+/− (weak)	+/− (weak)	+/− (weak)
50°C	+	−	−	−	−	−	−	−	−
Maximum NaCl concentration for growth	10%	5%	6%	>18%	8%	8%	10%	10%	10%
Growth at pH 4.5	+	+	+/−	−	+	+	+	+	−
pH 5.0	+	+	+	−	+	+	+	+	−
pH 7.5	+	−	+	+	+/−	+/−	+	+	+
pH 8.0	+	−	−	+	−	−	+	+	+
pH 8.5	+/−	−	−	+	−	−	+/−	+/−	+
Catalase activity	−	−	−	−	−	−	+/−	+/−	+/−
Gas from gluconate	−	−	+	−	−	−	−	−	−
Arginine hydrolysis	+	−	−	−	−	−	+	+	−
Hippurate hydrolysis	−	−	−	−	−	−	−	−	+
Production of acetoin	+/−	+/−	+/−	−	+/−	−	+/−	+/−	−
Lactate configuration	DL	DL	L(+)	L(+) (3% D(−))	DL	DL	DL	DL	L(+)
Litmus milk reaction									
Acid	+/−	−	+/−	−	+/−	−	+	+	ND
Reduction	+/−	−	+/−	−	+/−	−	+	+	ND
Clotting	+/−	−	−	−	−	−	+/−	+	ND
Acid produced from or splitting of									
Arabinose	+/−	−	−	+	−	−	+	−	+/−
Ribose	+	−	−	+	−	−	+	+	ND
Xylose	+	−	−	−	−	−	+/−	−	+/−
Fructose	+	+	+	+	+	+	+	+	+
Rhamnose	+/−	−	−	−	−	−	+/−	−	ND
Glucose	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+
Galactose	+	+/−	+	+/−	+	+/−	+	+	+
Maltose	−	+/−	+	+	+	+/−	+	+	+
Trehalose	+/−	+/−	+/−	+	+	+	+	+/−	+/−
Cellobiose	+	+	+	+	+	+	+	+	ND
Sucrose	+/−	+/−	+/−	+	−	−	−	+/−	+
Lactose	+/−	−	+/−	−	+	−	+	+	+/−
Melibiose	−	−	−	−	−	−	+/−	+/−	ND
Melezitose	−	+/−	−	+	−	−	−	−	ND
Raffinose	+/−	−	−	+/−	−	−	+/−	+/−	+
Maltotriose	−	+/−	+	+	+/−	+/−	+/−	+/−	ND
Dextrin	+/−	−	+	+/−	+/−	+/−	−	−	+/−
Starch	−	−	+	−	−	−	−	−	−
Inulin	−	−	+/−	−	−	−	+/−	+/−	−
Glycerol	+/−	−	−	+/−	−	−	+/−	+/−	−
Mannitol	+/−	−	−	−	−	−	−	−	+/−
Sorbitol	−	−	−	−	−	−	−	−	+/−
α-Methyl glucoside	−	+/−	+/−	+	+/−	−	−	−	ND
Salicin	+/−	+/−	+	+	+	+	+	+/−	+
Amygdalin	+/−	+/−	+	+	+	+/−	+	+/−	ND

+, >90% strains positive; +/−, 10–90% strains positive; −, <10% strains positive; ND, not determined.
†Characters in bold are useful for discrimination of species.

products other than lactate are formed. For example, *P. pentosaceus* produces an equimolar mixture of acetate and lactate from pentose sugars (Fukui *et al.*, 1957). Back (1978a) showed that the differences in lactate configuration result from the activities of different lactate dehydrogenases. Indeed, the electrophoretic mobility of such enzymes provides an aid to differentiation of *Pediococcus* species (see below). *Pediococcus pentosaceus* forms D(−) and L(+)-lactate from glucose but converts malic acid to L(+)-lactate (Radler *et al.*, 1970). The ability of *P. halophilus* to metabolize organic acids, such as citrate and malate, has been investigated by Kanbe and Uchida (1982, 1987b). The metabolism of citrate by this organism differs from that in lactic streptococci. Acetate and formate are the main products of citrate metabolism: no acetoin or diacetyl are produced (Kanbe and Uchida, 1987b). Many strains of *P. damnosus* form diacetyl (Shimwell and Kirkpatrick, 1939).

All strains of *P. dextrinicus* grow on starch. It is not known whether starch breakdown occurs as a result of α-amylase, glucoamylase or other enzymic activities, or whether the extracellular enzymes involved are secreted into the growth medium, or located at the cell surface.

Pediococci oxidize some substrates. For example, *P. pentosaceus* uses glycerol when O_2 is available, producing lactic acid, acetic acid, acetoin and CO_2 (Dobrogosz and Stone, 1962a). Similarly, this organism can oxidize lactate to acetate and CO_2 (Thomas *et al.*, 1985).

5.3.2 *Nitrogen metabolism*

Pediococci grow best in rich media. Most strains need a range of amino acids including alanine, aspartic acid, glutamic acid, arginine, histidine, isoleucine, phenylalanine, proline, threonine, tyrosine, valine, tryptophan, cystine, glycine and leucine. Some strains need lysine, methionine and serine (Jensen and Seeley, 1954; Sakaguchi, 1960). Many strains grow poorly, or not at all, without a complex source of nitrogen such as peptides (Nakagawa and Kitahara, 1959). Aminopeptidases are produced by some strains. For example, Tzanetakis and Litopolou-Tzanetaki (1989), using the API ZYM test kit, showed that *P. pentosaceus* produces leucine aminopeptidase and valine aminopeptidase.

Bhowmik and Marth (1990b) examined the intracellular protease, endopeptidase, dipeptidase, dipeptidyl aminopeptidase and carboxypeptidase activities of six strains of *P. pentosaceus* and two strains of *P. acidilactici*. All the strains produced proteases, dipeptidases, dipeptidyl aminopeptidases and aminopeptidases, but did not produce carboxypeptidases or endopeptidases. Crude cell-free extracts of most strains partially hydrolysed α_{s1}-casein. *Pediococcus pentosaceus* ATCC 996 completely hydrolysed this protein. β-Casein was completely hydrolysed by some strains, but only partially by others.

Little information is available about the proteolytic activities of other pediococci, with the exception of a report by Davis *et al.* (1988) indicating that *P. parvulus* does not possess proteolytic activity, and one by Uhl and Kühbeck (1969) indicating that growth of *P. damnosus* in beer is associated with endo-enzymic hydrolysis of short peptides and exo-enzymic hydrolysis of polypeptides.

5.3.3 *Vitamin and organic base requirements*

All species need nicotinic acid, pantothenic acid and biotin for growth. Thiamine, *p*-aminobenzoic acid and cobalamin are not essential. Some strains need riboflavin, pyridoxine and folinic acid (Sakaguchi and Mori, 1969). Pyridoxin stimulates growth of most strains of *P. damnosus* and is essential for growth of some (Solberg and Clausen, 1973b). Most do not need preformed organic bases. Adenine, guanine, uracil and xanthine do not stimulate growth of pediococci in a defined medium (Sakaguchi and Mori, 1969).

An interesting coda relating to the vitamin requirements of pediococci concerns the case of '*Leuconostoc citrovorum*' strain 8081. Dunn *et al.* (1947) first studied the nutrition of this organism and found that, in addition to a requirement for 16 amino acids, a 'concentrate' of folic acid was required for optimal growth. Sauberlich and Baumann (1948) showed that the strain needed a growth factor, found in liver extract, which they named 'citrovorum factor'. High concentrations of folic acid could replace this unknown factor, thus explaining why Dunn *et al.* (1947) had not been aware of the requirement. The substance was later identified and referred to both as folinic acid-SF and leucovorin. It is now known as 5-formyltetrahydrofolic acid (5-formyl-THF). ('Citrovorum factor' is sometimes incorrectly referred to as folic acid (e.g. Raccach, 1987).) Confusion temporarily arose when it was discovered that the requirement for 5-formyl-THF was not generally found among other leuconostocs and that all pediococci which had been studied up to that time needed the factor for growth. Further studies showed that the requirement was restricted only to some strains of pediococci (Günther and White, 1961a). The paradox was resolved when it was shown that '*Leuconostoc citrovorum*' strain 8081 was not a leuconostoc at all, but belonged to the pediococci (Felton and Niven, 1953). Initially, it was named '*P. cerevisiae*' but would now be classified as *P. pentosaceus*.

Tetrahydrofolate derivatives usually play a metabolic role in transfer of single-carbon units. The significance of 5-formyl-THF in bacterial metabolism is not presently clear. However, the compound has been used in the treatment of malignant tumours in mammals, including man (Metzler, 1977).

5.3.4 *Mineral requirements*

All pediococci studied so far (*P. acidilactici*, *P. pentosaceus*) need large quantities of manganese for growth, and in this respect differ from *Enterococcus* spp. (Efthymiou and Joseph, 1972). In common with *Lactobacillus* spp., they have no requirement for iron (Archibald, 1986). Whether this is true of *P. halophilus* and *P. urinae-equi* is not known. Raccach (1981) studied the ability of different metal ions to stimulate the fermentative activity of *P. pentosaceus*. Stimulation followed the sequence $Mn^{2+} > Ca^{2+} > Fe^{2+} > Zn^{2+} = Fe^{3+} > Mg^{2+}$.

5.3.5 *Reaction to oxygen*

Pediococci are aero-tolerant anaerobes. Growth of some strains is improved by anaerobic incubation, particularly on primary isolation. Most pediococci are unable to control the redox potential (rH) of the growth medium (Nakagawa and Kitahara, 1959). *Pediococcus halophilus*, however, is exceptional in this respect. Kanbe and Uchida (1987a) correlated the ability of different strains of *P. halophilus* to control rH with possession of an NADH dehydrogenase. This species also produces pyruvate oxidase, which catalyses a direct reaction between pyruvate and molecular oxygen (Kanbe and Uchida, 1985).

In general, pediococci are catalase-negative, but some strains of *P. pentosaceus* produce a 'pseudo-catalase' which gives false positive reactions when the cells are tested with H_2O_2 (Felton *et al.*, 1953; Whittenbury, 1964). Forty-nine out of 75 strains of *P. pentosaceus*, isolated by Tzanetakis and Litopolou-Tzanetaki (1989) from raw goat's milk and Feta and Kaseri cheeses, gave weak catalase reactions. 'Pseudo-catalase' differs from true catalase in that it is insensitive to inhibition by azide and does not contain a haem group. Cells grown on media with a low glucose content are most likely to produce pseudo-catalase. *Pediococcus acidilactici* forms catalase when provided with haemin (Whittenbury, 1964). In spite of reports suggesting that cytochromes are produced by pediococci (Jensen and Seeley, 1954; Whittenbury, 1964), it is now accepted that this is not the case (Garvie, 1986a). Dobrogosz and Stone (1962a,b) suggest that a flavoprotein enzyme system donates electrons to oxygen, resulting in H_2O_2 formation. For example, an α-glycerophosphate oxidase has been identified in cells of *P. pentosaceus* (Dobrogosz and Stone, 1962a).

Pediococci do not possess a superoxide dismutase. Instead they protect themselves against damage by oxygen radicals using high concentrations of Mn(II) (Archibald, 1986).

5.3.6 *Cell wall chemistry*

The cell walls of pediococci have interpeptide bridges of the L-Lys-L-Asp type between the alanine and lysine residues. They have D-Asp linkages between positions three and four of the two peptide bridges (Kandler, 1970). However, *P. urinae-equi*, like *Aerococcus viridans*, has only one type of peptidoglycan polypeptide, with no interpeptide bridge. In these organisms the D-Ala carboxyl residue binds to the amino group of the adjacent L-Lys (Bergan *et al.*, 1984). Pediococci do not have techoic acids in their cell walls (Garvie, 1986a).

5.3.7 *Miscellaneous metabolic features*

Pediococci neither reduce nitrate nor produce indole from tryptophan (Nakagawa and Kitahara, 1959). In general, they do not hydrolyse hippurate, although some strains belonging to *P. urinae-equi* can (Tanasupawat and Daengsubha, 1983). Two species (*P. acidilactici*, *P. pentosaceus*) produce ammonia from arginine.

Lipase activity is generally weak, or absent, in pediococci (Davis *et al.*, 1988; Tzanetakis and Litopolou-Tzanetaki, 1989). Some strains of *P. damnosus* need mevalonic acid for growth (Kitahara and Nakagawa, 1958), while others have a requirement for CO_2 (Nakagawa and Kitahara, 1959).

Pediococci differ in their tolerance to NaCl. Strains of the salt-tolerant species *P. halophilus* grow in the presence of >18% NaCl. Other species are less tolerant; their sensitivity varies with the composition of the growth medium and conditions of incubation (Nakagawa and Kitahara, 1959; Coster and White, 1964).

Some species (e.g. *P. damnosus*, *P. inopinatus*, *P. parvulus*) are tolerant to ethanol as evidenced by their ability to grow in alcoholic beverages, such as beer, wine and cider. For example, all 23 strains of *P. parvulus* isolated from wine by Davis *et al.* (1988) could grow in the presence of 12.5% (w/v) ethanol, while five of the strains could grow in the presence of 15% ethanol.

Some strains of *P. damnosus* are eight- to 20-fold more resistant than sensitive strains to the antibacterial action of hop bitter acids and are thus better equipped to grow in hopped beer (Simpson and Fernandez, 1992).

5.4 Genetic features

The genus *Pediococcus* is genetically heterogeneous. Mol% G+C values of *Pediococcus* spp., determined by various methods, lie in the range 34–44

(see species descriptions for individual values – Sakaguchi and Mori, 1969; Kocur *et al.*, 1971; Solberg and Clausen, 1973a; Back, 1978a). Even if *P. urinae-equi* and *P. halophilus* are excluded from the genus, as suggested by some (Bergan *et al.*, 1984; Collins *et al.*, 1990); the range is similarly broad (35–44%).

Some strains harbour plasmids which range in size from 4.5–40 MDa (Graham and McKay, 1985; Torriani *et al.*, 1987). Some code for production of bacteriocins (see below), others for fermentation of carbohydrates. In *P. pentosaceus*, the ability to ferment raffinose, melibiose and sucrose is associated with three different plasmids. Sucrose hydrolase and α-galactosidase activities are associated with plasmid-encoded raffinose utilization (Gonzalez and Kunka, 1986). Lactose fermentation in some strains of *P. pentosaceus*, and sucrose fermentation in some strains of *P. acidilactici*, may be plasmid-linked (Hoover *et al.*, 1988). Kayahara *et al.* (1989) found that 92 of 160 strains of *P. halophilus*, isolated from miso and soya sauce factories, harboured plasmids. In *P. acidilactici*, erythromycin resistance is coded for by a 40 MDa plasmid (Torriani *et al.*, 1987).

Pediococci can be transformed by electroporation or conjugation (Kim *et al.*, 1992). Plasmids can be transferred from genera such as *Enterococcus*, *Streptococcus* and *Lactococcus* to *Pediococcus* spp. and vice versa (Gonzalez and Kunka, 1983).

Bacteriophage attack of *P. halophilus* has been observed (Uchida and Kanbe, 1993) but phages that attack other pediococci are not known. Those attacking *P. halophilus* have a narrow host spectrum and several phage types of this species can be discriminated. This is consistent with the observation that *P. halophilus* is a heterogeneous species, consisting of many biovars which can be discriminated on the basis of their carbohydrate utilization patterns (Uchida, 1982).

5.5 Immunochemistry

Common precipitins are associated with *P. damnosus*, *P. parvulus* and '*P. cerevisiae*' (*P. pentosaceus* and *P. acidilactici*), but not with *P. halophilus* (Günther and White, 1961b). Antisera prepared against pediococci do not react with extracts prepared from closely related genera such as *Streptococcus* spp. and *Leuconostoc* spp. Coster and White (1964) found that antisera prepared against *P. parvulus* and *P. damnosus* reacted against '*P. cerevisiae*' (*P. pentosaceus*). Extracts of *P. halophilus* strains did not react with antisera prepared against other pediococci except for that prepared from one strain of '*P. cerevisiae*' (Coster and White, 1964). Antisera prepared against Coster and White's Group III strains (now classified as *P. dextrinicus*) showed cross-reactions with '*P. cerevisiae*', *P. parvulus* and some *P. damnosus* strains. Group III extracts gave no cross-reactions with antisera prepared against other pediococci.

London and co-workers used antibodies raised to aldolase enzymes to elucidate phylogenetic relationships among lactic acid bacteria, including *Pediococcus* spp. (London *et al.*, 1975; London and Chace, 1976, 1983).

Bhunia and Johnson (1992b) prepared monoclonal antibodies to several bacteriocin-producing strains of *P. acidilactici*. These antibodies did not react to other lactic acid bacteria or to other Gram-positive or Gram-negative organisms. A protein of M_r 116 000, located on the surface of *P. acidilactici* cells, was the antigenically reactive site (Bhunia and Johnson, 1992b). No reactions were obtained with proteins of identical M_r situated on the surfaces of *P. pentosaceus* cells, indicating that a specific epitope on the protein was responsible for antigenicity.

5.6 Historical aspects

Historical aspects of the genus *Pediococcus* have been thoroughly dealt with by a number of workers (Shimwell and Kirkpatrick, 1939; Shimwell, 1949; Pederson *et al.*, 1954; Garvie, 1974; Eschenbecher and Back, 1976). Salient features are summarized in Table 5.3.

Certain aspects of the literature relating to pediococci can be confusing, since the use of species names has lacked consistency. In particular, the name '*P. cerevisiae*', first used by Balcke (1884) for beer-spoilage strains (probably *P. damnosus*) that had only been observed microscopically and not isolated in pure culture, was used for plant pediococci (*P. pentosaceus*, *P. acidilactici*) by Pederson (1949). (Pederson mistakenly believed that plant pediococci and beer pediococci were one and the same.) Nakagawa and Kitahara (1959) used the name '*P. cerevisiae*' for beer-spoilage pediococci. In the literature spanning the 1960s, and to some extent even to the present day, this name has been applied to both groups of organisms. Günther *et al.* (1962) had proposed that ATCC 8081 be designated as the type strain of '*P. cerevisiae*' Balcke (this organism was originally known as '*Leuc. citrovorum*', see above). However, the description of this plant pediococcus was inconsistent with that described by Balcke (1884). As a result of a request from Garvie (1974), the Judicial Committee of the International Committee on Systematic Bacteriology issued an opinion in 1976 to the effect that the type species of the genus should be *P. damnosus* (Claussen, 1903) and the neotype strain Be.1 (NCDO 1832) (Judicial Commission, 1976). This strain was, in fact, isolated from lager beer by D.H. Williamson in our laboratories (then the Brewing Industry Research Foundation) more than 50 years ago. The name '*P. cerevisiae*' is no longer used.

The relationship between acidophilic beer-spoilage tetrad-forming cocci

Table 5.3 Historical development of the genus *Pediococcus**

Organism name	Reference	Comment
'Beer sarcinae'	Hansen (1879)	Produce 'sarcina sickness' in beer.
'*Pediococcus cerevisae*'	Balcke (1884)	Cell division in one plane, successive cell divisions at 90° to each other. Acid in sugar-containing media. Grow in beer. Optimum growth temperature 20–25°C.
Pediococcus acidi-lactici	Lindner (1888)	Optimum growth temperature 41°C. Produce large amounts of lactic acid in sugar-containing media.
'*Pediococcus sarcinaeformis*'	Reichard (1894)	Tetrad-forming coccus isolated from beer. Optimal growth temperature 20–25°C. Cells form clusters, or packets, under acidic conditions.
Pediococcus damnosus	Claussen (1903)	Cells grow in wort and pasteurized beer. Resistant to fluoride.
'*Pediococcus perniciousus*'	Claussen (1903)	Similar to *P. damnosus* but cells smaller. More vigorous growth in beer than *P. damnosus*.
'*Pediococcus henneberg*i'	Sollied (1903)	Optimal growth temperature 40°C. Maltose, galactose, glucose, arabinose and xylose fermented to give optically-inactive lactic acid. Differs from *P. acidilactici* in ability to ferment sucrose and arabinose.
'*Sarcina hamaguchiae*'	Saito (1907)	Salt-tolerant lactic acid-producing tetrad-forming coccus isolated from Japanese soya sauce mash.
'*Pediococcus damnosus* var. *perniciousus*'	Mees (1934)	Tetrad-forming cocci that produce DL-lactate from glucose.
'*Pediococcus damnosus* var. *salicinaceus*'	Mees (1934)	Similar to '*P. damnosus* var. *perniciousus*' but ferments salicin.
Pediococcus pentosaceus	Mees (1934)	Ferments arabinose. Grows at 45°C.
Pediococcus halophilus	Mees (1934)	Salt-tolerant tetrad-forming cocci.
Pediococcus urinae-equi	Mees (1934)	Produces less lactic acid than other pediococci and grows at alkaline pH values.
'*Streptococcus damnosus*'	Shimwell and Kirkpatrick (1939)	Synonym of *P. damnosus*.
'*Streptococcus tetragenus*'	Walters (1940)	Pentose-fermenting tetrad-forming cocci isolated from beer.
'*Streptococcus damnosus* var. *diastaticus*'	Andrews and Gilliland (1952)	Dextrin-degrading tetrad-forming cocci isolated from beer. Impart a bitter flavour and strong bitter after-flavour to beer.
'*Pediococcus mevalovorus*'	Kitahara and Nakagawa (1958)	Tetrad-forming cocci resembling *P. damnosus* but which require mevalonic acid for growth.
Pediococcus parvulus	Günther and White (1961a)	Tetrad-forming coccus which formed very small colonies on media used for isolation. (Later shown that growth could be improved by addition of Tween 80.) Serologically distinct from other pediococci.
Pediococcus damnosus var. '*damnosus*' var. '*diastaticus*' var. '*limosus*'	Coster and White (1964)	Proposed subspecies of *P. damnosus*.
Pediococcus inopinatus	Back (1978a)	New species of beer-spoiling pediococci. Identified, in addition to phenotypic behaviour, on the basis of DNA–DNA homology tests and electrophoretic behaviour of LDHs.
'*Pediococcus pentosaceus* var. *intermedius*'	Back (1978a)	Subspecies of *P. pentosaceus* identified on the basis of inability to use certain pentose sugars, DNA/DNA homology tests and electrophoretic mobility of LDHs.
Pediococcus dextrinicus	Back (1978b)	Valid publication of description of starch-degrading pediococci.
'*Tetratogenococcus halophilus*'	Collins *et al.* (1990)	Proposed new genus and species to accommodate strains described as *P. halophilus*.

*Note: This table is not comprehensive. For further details on the history of the development of the genus *Pediococcus* see Shimwell and Kirkpatrick (1939); Shimwell (1949); Garvie (1974); Eschenbecher and Back (1976).

and their acid-sensitive counterparts (*P. urinae-equi*) has long been the subject of debate and confusion. Indeed, many references can be found in the early brewing science literature to the relationship between organisms able to grow in horse urine and those able to grow in beer. Until the 1930s, most beer was transported by horse. Consequently, stables were maintained within the confines of the brewery. In finding that many beers suffered spoilage by tetrad-forming cocci, many brewery bacteriologists searched for the source of such contaminants. They found brewery stables to be heavily contaminated with such bacteria, and the urine of horses to be a particularly good source. However, it remained unknown whether the cocci that could be isolated from this source could spoil beer. A large number of studies were carried out in breweries to establish a link.

Typical of such studies was that of Stockhausen and Stege (1925) who showed that 'sarcinae' isolated from horse urine could grow in pasteurized beer and produce the 'usual symptoms' of sarcina sickness after 3–4 weeks at 25°C. Conversely, they showed that 'sarcinae' isolated from beer developed in sterile horse urine, of neutral pH value, forming a cloudiness after 6–9 days incubation. As a result of these studies they concluded that 'urinary sarcinae, which originally find their most favourable habitat in an alkaline medium, are capable, without gradual adaptation, of producing sarcina sickness in beer, a point of practical importance in regard to the location of stables in breweries'. In light of current knowledge of pediococci, this finding seems most likely to have been caused by use of mixed cultures rather than by adaptation of a pure culture.

Back (1978a) made a substantial taxonomic study of the genus, isolating 840 pediococcus colonies from a range of sources. He identified them on the basis of biochemical characteristics and genetic attributes. In addition to confirming the known species of *P. pentosaceus*, *P. acidilactici*, *P. parvulus*, *P. damnosus* and *P. halophilus*, Back identified two further species for which he proposed the names *P. inopinatus* (Back, 1978a) and *P. dextrinicus* (Back, 1978b). In addition, he found that some strains, which had been isolated from plant materials and had been identified as *P. damnosus* on the basis of biochemical attributes (in particular, their inability to use pentoses) had a high level of genetic homology with *P. pentosaceus*. He proposed that these organisms were closely related to *P. pentosaceus*, but distinct from *P. damnosus*, and named them '*P. pentosaceus* subsp. *intermedius*' (Back, 1978a).

5.7 Phylogenetic relationships

DNA–DNA homology assays (Back and Stackebrandt, 1978; Dellaglio *et al.*, 1981; Dellaglio and Torriani, 1986) have been used to elucidate phylogenetic relationships among pediococci. Table 5.4 shows the

homology values obtained for representatives of each species. These support the groupings made on the basis of physiological tests. For example, although *P. pentosaceus* and *P. acidilactici* can sometimes be difficult to separate using phenotypic tests, DNA–DNA homology assays reveal only 5–35% homology, thus justifying separation of the species (Back and Stackebrandt, 1978; Dellaglio and Torriani, 1986). *Pediococcus damnosus* shows a significant degree of homology with *P. inopinatus* and *P. parvulus* (41–54 and 34–36%, respectively), but little homology to other species. *Pediococcus dextrinicus* has little genetic homology with the other pediococci (0–8%). Likewise, *P. urinae-equi* had no detectable homology with other members of the genus. Although they differ with respect to their phenotypic properties, strains belonging to *P. pentosaceus* and '*P. pentosaceus* subsp. *intermedius*' are clearly related as evidenced by homology values of 88–97%. Dellaglio and Torriani (1986) isolated three strains of pediococci from maize silage that resembled *P. pentosaceus* phenotypically but did not show significant DNA homology with strains belonging to this species. These isolates remain unidentified.

Stackebrandt *et al.* (1983), on the basis of 16S rRNA oligonucleotide cataloguing, suggested that *Pediococcus* spp. and *Leuconostoc* spp. were phylogenetically related to lactobacilli but distinct from streptococci. In this respect, they confirmed earlier immunological studies reported by London and Chace (1976). They suggested that descriptions of the genus *Lactobacillus* should be extended to include cocci occurring in pairs and chains (Stackebrandt *et al.*, 1983). This suggestion has not been generally accepted. The genus *Pediococcus* is placed within the Gram-positive cocci in the ninth edition of *Bergey's Manual* published in 1986. Kandler and Weiss (1986) stated that more work was needed concerning the phylogenetic relationships between the genus *Lactobacillus* and other lactic acid bacteria before the suggestion of Stackebrandt *et al.* could be considered.

Phylogenetic relationships between members of the genus *Pediococcus* and other genera including *Lactobacillus*, *Enterococcus*, *Vagococcus*, *Carnobacterium* and *Aerococcus*, were explored by Collins *et al.* (1990) who analysed 16S rRNA sequences. Calculation of sequence homologies allowed the species to be compared (Table 5.5). These values showed that the genus *Pediococcus*, as presently constituted, was phylogenetically heterogeneous. *Pediococcus acidilactici*, *P. damnosus*, *P. parvulus* and *P. pentosaceus* formed a distinct group, but *P. halophilus* had little homology with other pediococci. Homology values comparing *P. halophilus* and other pediococci (with the exception of *P. urinae-equi*) did not exceed 89.7%. In fact, *P. halophilus* had a closer affinity with members of the genus *Enterococcus* and *Carnobacterium* than with members of the genus *Pediococcus* or with other lactic acid bacteria. Collins *et al.* (1990) suggested that this organism be reclassified in a new genus and named '*Tetratogenococcus halophilus*'. *Pediococcus urinae-equi* was found to be

Table 5.4 DNA–DNA homology among pediococci*

	P. acidilactici	*P. damnosus*	*P. dextrinicus*	*P. inopinatus*	*P. halophilus*	*P. parvulus*	*P. pento-saceus*	*P. pento-saceus* subsp. *intermedius*	*P. urinae-equi*
P. acidilactici	100								
P. damnosus	0–7	100							
P. dextrinicus	0–5	4–5	100						
P. inopinatus	0–7	41–54	7	100					
P. halophilus	0–2	0–2	6	3–5	100				
P. parvulus	0–7	34–36	8	30–40	4	100			
P. pentosaceus	5–35	0–18	6	7–8	4	7	100		
'*P. pentosaceus* subsp. *intermedius*'	17–19	0–7	5	6–7	3	6	88–97	100	
'*P. urinae-equi*'	0	0	0	0	0	0	0	0	100

*Data compiled from Back and Stackebrandt (1978), Dellaglio *et al.* (1981), and Dellaglio and Torriani (1986).

Table 5.5 Percentage homology for a 1340-nucleotide region of 16S rRNAs of *Pediococcus* spp. and *Aerococcus viridans**

	P. acidilactici NCDO 2767	*P. damnosus* NCDO 1832	*P. dextrinicus* NCDO 1561	*P. halophilus* NCIB 12011	*P. parvulus* NCDO 1634	*P. pentosaceus* NCDO 990	*P. urinae-equi* NCDO 1636	*A. viridans* NCDO 1225
P. acidilactici NCDO 2767	100							
P. damnosus NCDO 1832	96.6	100						
P. dextrinicus NCDO 1561	93.8	94.0	100					
P. halophilus NCIB 12011	89.7	88.7	88.6	100				
P. parvulus NCDO 1634	97.0	98.7	94.5	87.4	100			
P. pentosaceus NCDO 990	98.3	96.5	93.2	88.3	96.7	100		
P. urinae-equi NCDO 1636	90.3	89.3	90.5	90.4	89.8	89.6	100	
A. viridans NCIDO 1225	89.3	89.9	89.6	89.7	89.6	89.0	99.9	100

*Data from Collins *et al.* (1990).

very closely related to *A. viridans* (99.9% sequence homology) confirming the view of Sakaguchi and Mori (1969) that the organisms belonged to the same species. The relationships between pediococci and other lactic acid bacteria, highlighted by 16S rRNA cataloguing, are summarized in the unrooted phylogenetic tree shown in Figure 5.3.

Many of these findings are supported by other chemotaxonomic studies. Thus, pediococci (with the exception of *P. urinae-equi*) differ from members of the genus *Leuconostoc* in that the former contain aspartic acid residues in their cell walls while the latter do not (Kandler, 1970). The cellular fatty acid spectra of *Pediococcus* spp. can be used to discriminate such organisms from those belonging to other genera and to differentiate between members of the genus (Uchida and Mogi, 1972; Bergan *et al.*, 1984). Bergan *et al.* (1984) divided the pediococci into three groups on the basis of their fatty acid composition. Group I, containing *P. urinae-equi* (which they considered identical to *A. viridans*), was characterized by the presence of C:14,1 and C:12 fatty acids. An unidentified peak, representing a fatty acid containing 12 or 13 carbon atoms, was also detected. This peak was not present in any other group. Group II strains (*P. damnosus*, *P. dextrinicus*, *P. halophilus*, *P. inopinatus* and *P. parvulus*) contained significant quantities of cyclopropane fatty acids (C:17cy; C:19cy). These were absent from other pediococci. Strains belonging to Group III (*P. acidilactici*, *P. pentosaceus* and '*P. pentosaceus* subsp. *intermedius*') lacked all of the fatty acids characteristic of Groups I and II, viz. C:12, C:14, C:17cy and C:19cy. However, they contained a unique small peak, probably a C:15 fatty acid, and had a much lower content of C:14 fatty acids than strains belonging to other groups. *Pediococcus dextrinicus*

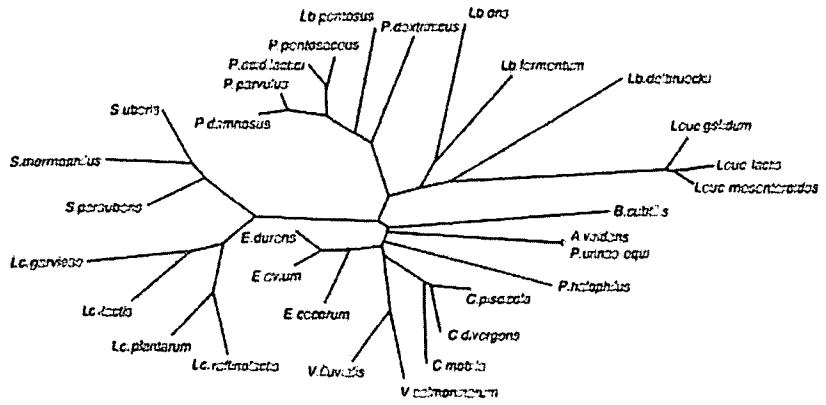


Figure 5.3 Unrooted phylogenetic tree showing the relationship between pediococci and other species. (From Collins *et al.* (1990), reproduced by permission of the Federation of European Microbiological Societies).

differed from other Group III strains in that it contained a higher concentration of C:18,1 and no C:19 fatty acids. A similar division of the pediococci based on fatty acid composition has also been described by Uchida and Mogi (1972).

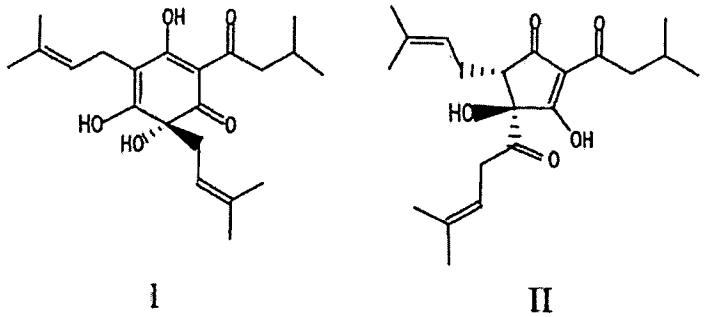
5.8 Importance of the genus

5.8.1 Beer spoilage

Only two species, *P. damnosus* and *P. inopinatus*, grow in beer, but not all strains belonging to these species are able to do so. Others merely survive, or grow only in those of low alcohol content, high pH value, or low content of hop bitter acids (Back, 1978a; Lawrence and Priest, 1981).

The ability of an isolate to grow in beer has been given substantial taxonomic importance in the past. However, the beers used to determine the result of this test have varied considerably. They have included a French 'country beer' (Bière de garde) (Claussen, 1903), lager beer of the Pilsen type (Hansen, 1879; Back, 1978a) and stout (Andrews and Gilliland, 1952). Beers differ in their ability to support growth of pediococci (Dolezil and Kirsop, 1980). Fermentable carbohydrate, amino nitrogen (in the form of both amino acids and peptides), some vitamins, and minerals such as manganese, are limiting to growth (Uhl and Kühbeck, 1969; Rainbow, 1981). Beer has a relatively low pH value (typically 3.8–4.6) and contains ethanol and CO_2. Perhaps the most significant factor affecting the ability of pediococci to grow in beer is the presence of antibacterial compounds derived from hops (Figure 5.4). The antibacterial activity of these compounds is pH-dependent, being greater at low pH (Simpson and Smith, 1992). Thus, in tests designed to compare the sensitivity of different strains to such compounds, the pH value of the medium must be carefully controlled and reported alongside the results. At pH 5.2 in modified MRS, strains of *P. damnosus* which are resistant to hop bitter acids have minimum inhibitory concentrations (MIC) of about 100 μM, while sensitive strains, including those belonging to *P. pentosaceus*, have MIC of 20 μM or less (Fernandez and Simpson, 1993). Most beer spoilage lactic acid bacteria are unable to grow in beer unless they are grown under specialized conditions. For example, growth of the cells in the presence of a subinhibitory concentration of iso-α-acids, before inoculation into beer stimulates their ability to grow in beer (Simpson and Fernandez, 1992). The ability of different species or strains to grow in 'beer' is clearly an unreliable characteristic unless care is taken to describe the conditions of pre-culture and the chemical composition of the beer.

Pediococci are ubiquitous contaminants of breweries. *Pediococcus acidilactici* and *P. pentosaceus* are found on malt and can grow during the



I II

Figure 5.4 Hop bitter acids. α-Acids such as (−)-humulone (I) are found in hops; iso-α-acids, such as *trans*-isohumulone (II) are found in beer. Both are inhibitory to Gram-positive bacteria but many strains of *P. damnosus* are relatively resistant to both.

early stages of wort production when the temperature is of the order of 50°C and no hop compounds are present. Growth of these bacteria is not known to cause any defect in the beer produced from such worts. However, if the mash temperature is not controlled, growth of pediococci and thermophilic lactobacilli can result in acidification.

Strains belonging to *P. damnosus* and *P. inopinatus* can grow during fermentation and survive to be harvested with the yeast crop. For example, in this laboratory we have found *P. damnosus* at a level of up to 2×10^5 cfu/10^6 yeast cells in a contaminated commercial culture. The organisms were active during the fermentation process in the affected brewery, causing high levels of diacetyl to accumulate in the beer. More typically, such organisms are present at a level of 0–100 cfu/10^6 yeast cells.

McCaig and Weaver (1983) examined the physiological properties of a range of pediococci from a lager brewery. They isolated *P. damnosus* from yeast and throughout the fermentation process, in addition to the later stages of processing and in the final unpasteurized beer. Organisms which they referred to as *P. damnosus* 'var. 1' (probably *P. inopinatus*) could be isolated only from yeast and the early stages of fermentation. Occasionally, they were found in the later stages of fermentation, but never during conditioning or in unpasteurized beer. Those belonging to *P. pentosaceus* could be isolated from all stages and from final beer, but more commonly in the early stages of processing.

Rope (extracellular polymer) can be formed in beer by pediococci (Shimwell, 1948b; Kulka *et al.*, 1949). In some cases, rope production is so great that it is possible to draw the beer out into strings of almost 1 m in length without breaking them (Shimwell, 1948b). Nakagawa and Kitahara (1959) did not isolate any rope-forming strains of pediococci, but found that some strains produced rope when they were inoculated into media containing 0.05% agar.

Some strains now classified as *P. damnosus* (formerly '*P. mevalovorus*') require mevalonic acid for growth (Kitahara and Nakagawa, 1958). Mevalonic acid is not found in wort but is produced during the fermentation process. These strains can thus grow in beer, but are unable to grow in wort.

The source of *P. damnosus* contamination in breweries is frequently contaminated pitching yeast, beer, or equipment. Green and Gray (1949) isolated pediococci from the air of one brewery using an electrostatic sampler.

5.8.2 Wine and cider

Some pediococci produce off-flavours in wine, caused by diacetyl and acetoin (Pilone and Kunkee, 1965). Others spoil wine by producing rope, a D-glucan consisting of a trisaccharide repeating unit of D-glucose, that increases the viscosity of the wine to such an extent as to make it unpalatable (Llaubères *et al.*, 1990). Wines with higher than average pH values are more susceptible to growth of pediococci. Edwards and Jensen (1992) isolated nine strains of *P. parvulus* from wine and tentatively identified one strain as *P. inopinatus*.

Pediococci play a minor role in cider microbiology. Carr (1970) isolated several strains from spoiled ciders which he identified as '*P. cerevisiae*', some of which could form slime. These isolates can now be classified as *P. inopinatus* on the basis of their biochemical characteristics.

5.8.3 Soft drinks

Unidentified *Pediococcus* spp. were among the most prevalent of bacteria isolated from carbonated soft drinks in a Nigerian factory (Odunfa, 1987). Pediococci do not usually cause problems in soft drink production.

5.8.4 Soya sauce and miso

Citric acid is the major organic acid produced in the early stages of moromi fermentation, the second stage of the two-stage soya sauce process. Kanbe and Uchida (1987b) found that the ability of different strains of *P. halophilus* to utilize citrate correlated with production of the inducible enzyme, citrate lyase. The main products of citrate metabolism were acetate and formate; diacetyl was not formed.

Immobilized cells of *P. halophilus* can be used to prepare soya sauce. Osaki *et al.* (1985) described a two-stage process, in which *P. halophilus* cells were immobilized in one bioreactor and yeast cells immobilized in a second bioreactor. This protocol allowed the soya sauce production time to be reduced from 6 months to 2 weeks (Osaki *et al.*, 1985). Subsequently, *P.*

halophilus has been immobilized within porous alumina ceramic beads to produce a feedstock for yeast fermentation in soya sauce production (Iwasaki *et al.*, 1993).

Salt-tolerant pediococci also play a role in the fermentation of miso, a fermented food prepared from mould, rice, soyabeans and salt (Shibasaki and Hesseltine, 1962).

5.8.5 Cheese

The presence of pediococci in cheese was first reported by Dacre (1958a). Starter cultures of *Pediococcus* spp. have since been employed in cheese production (Dacre, 1958b; Bhowmik and Marth, 1990a). Pediococci represent only a small proportion of the total lactic acid bacteria in cheese (Litopolou-Tzanetaki *et al.*, 1989). Their precise influence on cheese quality is not yet fully understood (Bhowmik and Marth, 1990a; Fox *et al.*, 1990; Olson, 1990).

5.8.6 Meat and fish products

Starter cultures, consisting of a selected strain of *P. acidilactici*, are used in the preparation of semi-dry sausages. An ability to metabolize in conditions of reduced a_w favours pediococci in this matrix. Benefits of the use of such cultures include improvements in sausage uniformity and a reduction in process times (Everson *et al.*, 1970). Both lyophilized cultures (Deibel *et al.*, 1961) and frozen cell concentrates (Porubcan and Sellars, 1979) have been used. *Pediococcus pentosaceus* can be used instead of *P. acidilactici*. It is better suited to dry sausage fermentation as it has a lower optimal growth temperature and a lower minimum temperature for fermentation (Raccach, 1987).

Pediococci can be used to protect other types of sausage from pathogens, such as *Listeria monocytogenes*, since they produce bacteriocins (Berry *et al.*, 1991; Yousef *et al.*, 1991; Foegeding *et al.*, 1992; Luchansky *et al.*, 1992). Commercial starter cultures of *P. acidilactici* (e.g. 'Accel' from Muller in Germany) have been used to prevent meat spoilage (Gibbs, 1987).

Pediococci are present in various fermented foods including buroung dalag (a Philippino dish prepared from dalag fish and rice), marinated herrings (Blood, 1975), pla-som (Thai fermented fish), som-fak (Thai fish cake), nham (Thai fermented pork), fermented shrimps and a wide range of Thai fermented foods (Tanasupawat and Daengsubha, 1983).

5.8.7 Miscellaneous roles in fermentation processes

Costilow and Gerhardt (1983) used *P. pentosaceus* in a dialysis fermentation system to prepare fermented brined cucumbers and green beans. The

inoculum and vegetables were separated by a semi-permeable dialysis membrane. The process was unsatisfactory with respect to the rate of acid production, pH reduction and utilization of carbohydrates (Costilow and Gerhardt, 1983). Use of a mixed inoculum containing *Lactobacillus plantarum*, *Streptococcus faecium* and an unidentified pediococcus improved control of silage fermentation, reducing losses and restricting growth of undesirable organisms (Weinberg *et al.*, 1988). Fitzsimons *et al.* (1992) obtained similar results using pure cultures of *P. acidilactici*. This organism and *P. pentosaceus* also effect some of the microbiological transformations that occur in uninoculated silage (Langston and Bouma, 1960; Lin *et al.*, 1992). *Pediococcus pentosaceus* has been used to ferment heat-treated soya milk (Raccach, 1987). Tou-pan-chiang is a traditional Chinese fermented food. In a study of the microorganisms found in Tou-pan-chiang mash, Hwang *et al.* (1988) isolated 88 strains of lactic acid bacteria, 33 of which were salt-tolerant strains of pediococci. Strains of *P. pentosaceus* are also associated with some vegetable fermentations. Examples include pickled cucumbers (Etchells *et al.*, 1975), olives and sauerkraut (Stammer, 1975).

5.8.8 Pediocin production

Pediocins, bacteriocins produced by pediococci, have been discussed in Volume 1 of this series (Earnshaw, 1992; Vandevoorde *et al.*, 1992). They include pediocin AcH (Bhunia *et al.*, 1988; Biswas *et al.*, 1991), bacteriocin PA-1 (Gonzalez and Kunka, 1987; Pucci *et al.*, 1988) and pediocin SJ-1 (Schved *et al.*, 1993) produced by strains of *P. acidilactici*, pediocin A produced by *P. pentosaceus* (Fleming *et al.*, 1975; Daeschel and Klaenhammer, 1985) and an unnamed bacteriocin (Hoover *et al.*, 1988) produced by an unidentified *Pediococcus*. Pediocins can be separated on the basis of their sensitivity to different proteolytic enzymes, chromatographic behaviour and molecular structure (Ray, 1992a). A broad spectrum antibacterial activity produced by three strains of *P. damnosus* isolated from beer was studied by Skyttä *et al.* (1993). The antibacterial compounds inhibited growth of both Gram-positive and Gram-negative bacteria, were thermotolerant, non-proteinaceous, and thus atypical bacteriocins. Their identification awaits further work. Pediocins of *P. acidilactici* have been extensively reviewed (Ray, 1992a) as have those of *P. pentosaceus* (Daeschel, 1992).

Pediocin production is often plasmid-linked. For example, the ability of *P. acidilactici* to produce pediocin AcH is correlated to possession of an 11.1 kb plasmid (pSMB74): the plasmid does not encode pediocin resistance (Kim *et al.*, 1992; Ray *et al.*, 1992). Pediocin AcH is a basic polypeptide (p*I* 9.6) that contains 44 amino acids and two disulphide bonds. Post-translational modification cleaves 18 amino acids from a

'prepediocin' of 62 amino acids to form the active pediocin (Ray, 1992b). A microbiological overlay technique used after separation by SDS-PAGE can be used to identify it (Bhunia and Johnson, 1992a).

Marugg *et al.* (1992) cloned the genes responsible for production of pediocin PA-1, a 44 amino acid polypeptide, and expressed them in *Escherichia coli*. Production of pediocin PA-1 depends on the presence of four clustered open reading frames (*ped*A, *ped*B, *ped*C and *ped*D). The *ped*A gene encodes a 62 amino acid precursor of pediocin PA-1; the *ped*B and *ped*C genes encode proteins of 112 and 174 amino acids, respectively; their function is not known. The *ped*D gene may be involved in translocation of pediocin PA-1 and, possibly, also of pediocin AcH (Marugg *et al.*, 1992).

Bhunia *et al.* (1991) investigated the mode of action of pediocin AcH against sensitive bacteria. Following exposure to the bacteriocin, sensitive cells released K^+ and UV-absorbing materials into the medium and became more permeable to larger molecules such as *o*-nitrophenol-β-D-galactopyranoside. Binding of the bacteriocin molecules to specific receptors on the surface of sensitive bacteria preceded its bactericidal effects. Non-specific and specific receptors for pediocin AcH are present on the surface of sensitive cells (Bhunia *et al.*, 1991). In the case of mutant strains of Gram-positive bacteria which are resistant to pediocin AcH, the specific receptors may either be absent, or not available for binding. In the case of Gram-negative bacteria, which are insensitive to pediocin AcH, both non-specific and specific receptor sites are absent (Bhunia *et al.*, 1991).

Kalchayanand *et al.* (1992) showed that pediocin AcH-resistant Gram-negative and Gram-positive bacteria could be sensitized to the pediocin if they were first exposed to a sublethal stress, such as heat, freeze–thawing or acid treatment.

No pediocin has yet been approved for food use by any regulatory authority. However, a US patent has been granted that relates to use of pediocin PA-1 for prevention of spoilage of salad and salad dressings (Gonzalez, 1989). Ray (1992b) sees no reason why pediocins should not be approved for food use.

5.8.9 Biological assays

Pediococcus acidilactici NCIMB 6990 is used to assay pantothenic acid. A greater degree of accuracy can be achieved using this organism than when *Lactobacillus plantarum* is used (Solberg *et al.*, 1975).

5.8.10 Public health considerations

Pediococci are non-pathogenic. A few strains decarboxylate histidine to histamine, but most have low, or undetectable, activities of histidine decarboxylase and tyrosine decarboxylase (Radler, 1975). Biogenic amines can cause illness and Raccach (1987) suggested that pediococci should be tested for their ability to produce such compounds before using them to prepare foods. Bravo Abad (1990) showed that higher levels of biogenic amines are found in beers which have been contaminated with *Lactobacillus* spp. and *Pediococcus* spp. than in uncontaminated beers.

5.9 Isolation and enumeration of pediococci

A variety of media can be used to isolate pediococci. Because the genus is heterogeneous, no single medium or incubation conditions can be used for all species. For general purposes, MRS medium (de Man *et al.*, 1960), YGP medium (Garvie, 1978) and TGE medium (Biswas *et al.*, 1991) suffice. *Pediococcus halophilus* can be grown in YGP medium supplemented with 5% NaCl (Collins *et al.*, 1990). Tanasupawat and Daengsubha (1983) used GYP-calcium carbonate medium (pH 6.8), with or without 5% NaCl, to isolate a range of pediococci, including *P. urinae-equi* and *P. halophilus*, from foodstuffs. They screened for acid-forming colonies by checking for zones of clearing in the calcium carbonate, after aerobic incubation at 30°C. Nakagawa and Kitahara (1959) used end-fermented beer to which the pH value had been raised to 5.0 with sodium acetate, and which contained D-mannose as carbon source, to isolate pediococci from a variety of environments.

Nakagawa later developed a semi-solid agar that contains unhopped beer as a base (Nakagawa, 1970). Eto and Nakagawa (1975) isolated a strain of '*P. cerevisiae*' from beer which grew slowly on this medium. Enrichment with tomato juice extract or unhopped wort allowed good growth. A single substance, 4'-*o*-(β-D-glucopyranosyl)-D-pantothenic acid, was responsible for growth stimulation. Eto and Nakagawa (1975) recommended that 2% tomato juice should be added to media used to grow pediococci, or that, alternatively, the media should be dissolved in unhopped beer to meet the requirement of some strains for this component. In addition to providing the pantothenyl derivative required by some strains, tomato juice also stimulates growth of pediococci by providing manganese, together with complex nitrogen compounds, such as adenine and adenine derivatives.

Nakagawa (1964) also described a medium, based on unhopped beer, designed to detect beer-spoilage pediococci, including those strains that need mevalonic acid. In addition to unhopped beer, the medium contained mannose or salicin, a high concentration (20 g/litre) of sodium acetate, ascorbic acid, cycloheximide and agar.

Back (1978a) found that MRS medium supported good growth of most pediococci. However, 41 of 519 strains of *P. damnosus* isolated from beer,

beer yeast, breweries, wine and cider grew poorly (A_{578} 0.3–0.6). If the MRS was mixed 1:1 with lager beer, all strains grew well. Back speculated that this was related to a requirement of the strains for mevalonic acid. MRS medium supplemented with 4% NaCl, and adjusted to pH 7.0, was suitable for isolation of *P. halophilus* (Back, 1978a). In both media, most pediococci could be isolated at an incubation temperature of 28°C, except for beer-spoilage strains for which 22°C was more suitable (Back, 1978a).

Back (1978a) also successfully enriched cultures for certain species of pediococci by using melezitose, ribose or dextrin as carbon source, incubating the cultures at 50°C, or by including 10% NaCl to the growth medium.

Other media for detection of pediococci include NBB (Nachweismedium für bierschädliche Bakterien) (Dachs, 1981), VLB-S7 (Emeis, 1969), Raka–Ray agar no. 3 (Saha *et al.*, 1974), Hsu's rapid medium (Hsu *et al.*, 1975b) and Hsu's *Lactobacillus–Pediococcus* medium (Hsu *et al.*, 1975a), Lee's multidifferential agar (LMDA) (Lee *et al.*, 1975), sucrose agar (Boatwright and Kirsop, 1983) and KOT medium (Taguchi *et al.*, 1990). For *P. halophilus*, PAT agar (Uchida, 1982) or YGP broth supplemented with 5% NaCl can be used. The American Society of Brewing Chemists (1992) recommends the use of LMDA, Raka–Ray medium, Barney–Miller brewery medium and MRS agar for detection of brewery pediococci. The Institute of Brewing (1991) recommends a modified form of MRS and Raka–Ray medium for this purpose.

Carr (1970) used apple juice–yeast extract medium to isolate and maintain strains of '*P. cerevisiae*' (probably *P. inopinatus*) from spoiled cider.

On primary isolation, some pediococci are intolerant of oxygen; in addition, some have a requirement for CO_2. Commonly, cultures are incubated under anaerobic conditions. Anaerobic jars or cabinets, filled with a mixture of CO_2 and N_2, are suitable.

Pediococci can be isolated in the presence of lactobacilli by using MRS in which glucose has been replaced by 1% mannose, cellobiose or salicin (Back, 1978a). In the case of pediococci associated with plants (*P. acidilactici*, *P. pentosaceus*, '*P. pentosaceus* subsp. *intermedius*') incubation of primary cultures in Rogosa's SL medium at 45°C resulted in rapid initial growth of pediococci, with the result that they outgrew lactobacilli and other organisms that were present (Mundt *et al.*, 1969).

Selective agents used to assist isolation of pediococci from primary culture include cycloheximide and crystal violet to inhibit growth of yeasts and 2-phenylethanol, sorbic acid and acetic acid, to inhibit growth of both yeasts and Gram-negative bacteria. Thallous acetate inhibits growth of most microorganisms other than lactic acid bacteria (Sharpe, 1955). Vancomycin inhibits growth of Gram-positive bacteria other than those belonging to the genera *Pediococcus* and *Leuconostoc*, and some members

of the genus *Lactobacillus* (Simpson *et al.*, 1988). In the case of beer-spoilage strains of *P. damnosus*, hop bitter acids can be used as selective agents (Simpson and Hammond, 1991).

DNA probe techniques and immunochemical methods can be used to simultaneously identify and enumerate pediococci in test samples. For example, a monoclonal antibody colony immunoblot method, specific for bacteriocin-producing *P. acidilactici*, can detect such organisms in foods (Bhunia and Johnson, 1992b). DNA probe methods are available for detection and identification of glucan-forming wine-spoilage strains of *P. damnosus* (Lonvaud-Funel *et al.*, 1993), for non-glucan-forming strains of *P. damnosus* and *P. pentosaceus* in fermenting grape must and wine (Lonvaud-Funel *et al.*, 1991), and for strains of *P. pentosaceus* that colonize silage (Cocconcelli *et al.*, 1991).

Dolezil and Kirsop (1976) used a commercially available antiserum from Group D streptococci to detect *Pediococcus* spp. in brewers' yeast, wort and beer. Whiting *et al.* (1992) used an immunofluorescent antibody technique to detect diacetyl-producing pediococci in brewery pitching yeast.

Flow cytometry has been used to enumerate *P. damnosus* cells in brewery samples (Hutter, 1991). Interference from other microorganisms was minimized by using fluorescent dyes conjugated to antibodies (Hutter, 1992).

5.10 Maintenance and preservation of pediococci

Cultures of pediococci can be preserved in several ways. Most strains survive on agar slopes at 4°C provided they are sub-cultured regularly (usually every 3 months). The storage characteristics of the cultures are improved by addition of calcium carbonate (1%) to the growth medium to neutralize the acid produced by the organisms. Pediococci can also be preserved by lyophilization (Garvie, 1986a). Cells from a late logarithmic or early stationary phase should be suspended in horse serum, containing glucose (7.5%), prior to lyophilization. Alternatively, cells can be stored at −20°C in a mixture of growth medium and glycerol (1:1) (Weiss, 1991).

5.11 Identification of pediococci

Figure 5.5 shows a simple key to discrimination of *Pediococcus* spp. from other Gram-positive bacteria. This is based on production of catalase, anaerobic and aerobic growth, ability to produce gas from glucose, and cell morphology. Unlike other Gram-positive cocci (including *Streptococcus* spp., *Lactococcus* spp. and *Enterococcus* spp.), pediococci can grow in the

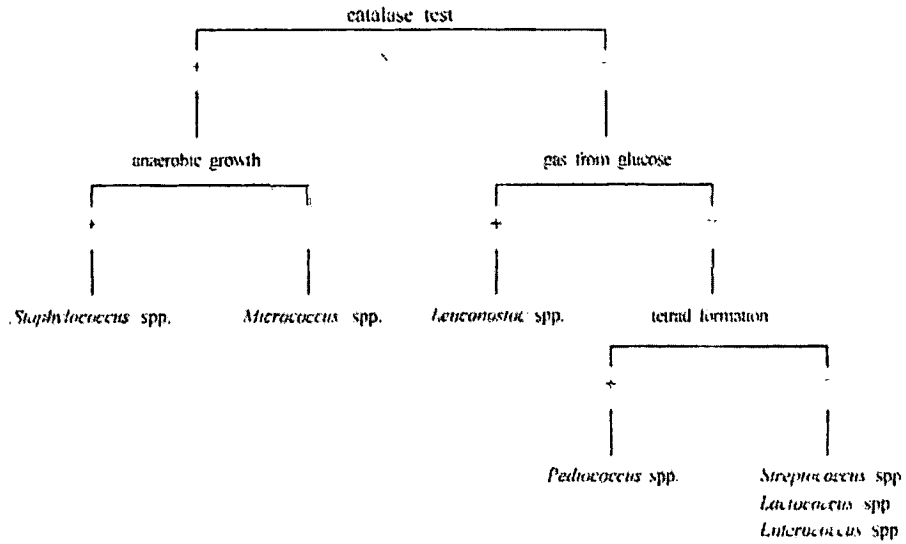


Figure 5.5 Key to differentiation of pediococi from other Gram-positive bacteria.

presence of vancomycin or avoparcin (50 mg/litre) (Simpson *et al.*, 1988). The biochemical basis of such resistance is not known. The reaction of *P. halophilus* and *P. urinae-equi* to vancomycin is not clear. Fourteen strains of *P. halophilus* isolated from porcine faeces were sensitive to vancomycin, but these strains were atypical with respect to several other phenotypic characters (Molitoris *et al.*, 1986). Pediococci can also be discriminated from lactococci by the fact that the former do not react to Group N streptococcal antiserum, while the latter give a positive reaction (Gonzalez and Kunka, 1983).

Pilone *et al.* (1991) described a single broth culture test, based on detection of heterofermentative metabolism, production of mannitol from fructose and production of ammonia from arginine, to differentiate between *Lactobacillus* spp., *Leuconostoc* spp. and *Pediococcus* spp. isolated from wine.

Figure 5.6 shows the key to identification of each of the eight *Pediococcus* species. Satisfactory differentiation can be achieved in most cases on the basis of growth at pH 8.5 and 4.2, at 40°C or 50°C, and in the presence or absence of 5% or 10% NaCl. Confirmatory tests, especially useful in the case of brewery pediococci, include those for acid from ribose, maltose, lactose or starch, hydrolysis of arginine, acid and gas from gluconate, and ability to grow at 35°C. Fuller details of differentiation between species of pediococci are given in the descriptions below.

An alternative scheme, proposed by Back (1978a), is shown in Figure

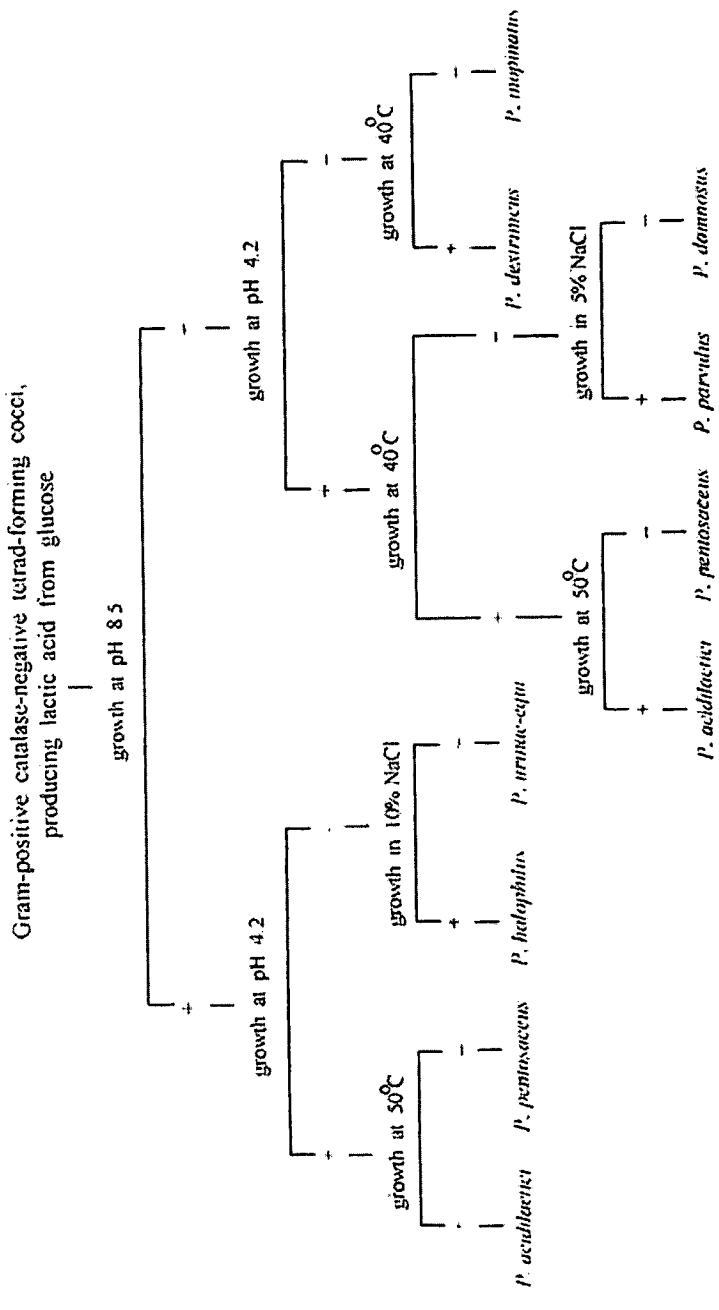


Figure 5.6 Key to the differentiation of the species of pediococci.

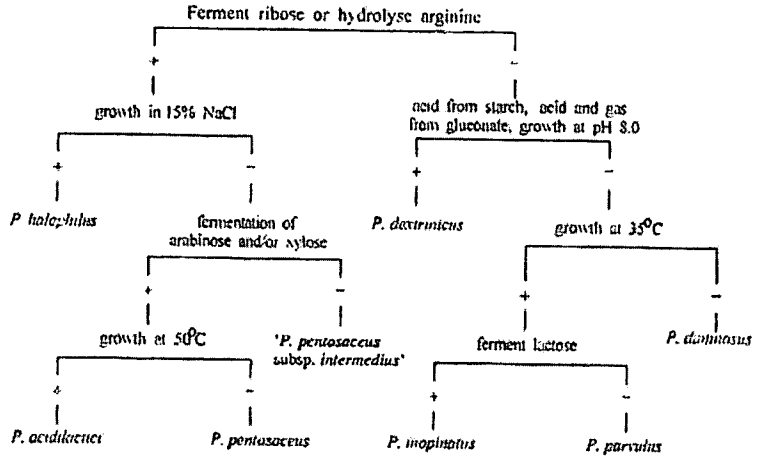


Figure 5.7 Alternative key to differentiation of pediococci. (Adapted from Back (1978a).)

5.7. Attributes in this scheme include the ability to hydrolyse arginine, fermentation of ribose, arabinose, xylose, lactose or starch, tolerance to 15% NaCl, ability to grow at 35°C or 50°C or at pH 8.0, and ability to produce acid and gas from gluconate. Confirmation of species identity can be obtained from sugar utilization profiles and from measurements of the electrophoretic mobility and characteristics of the cells' lactate dehydrogenase (LDH) (Back, 1978a). Pediococci produce three types of LDH: D-LDH and two types of L-LDH, one of which is activated by fructose-1,6-diphosphate. The electrophoretic mobilities of the different isozymes differ sufficiently to allow species differentiation (Table 5.6). DNA/DNA hybridization assays can also be used to confirm species identity (Back and Stackebrandt, 1978). Dolezil and Kirsop (1977) used the API lactobacillus system (now the API 50CH system) to discriminate between strains of pediococci. Lawrence and Priest (1981) confirmed the value of this kit for identification of brewery pediococci while Davis *et al.* (1988) used it to show the heterogeneity within the range of *P. parvulus* strains isolated from wine. Tzanetakis and Litopolou-Tzanetaki (1989) used the API ZYM test kit for biochemical characterization of 83 *P. pentosaceus* strains of dairy origin. Leucine aminopeptidase and valine aminopeptidase reactions were positive with all strains and of greatest intensity of the characters tested. β-Galactosidase and *N*-acetyl-β-glucosamidase were found in most strains. β-Glucosidase, esterase, esterase:lipase, lipase, phosphoamidase, cystine aminopeptidase and acid phosphatase activities were found in some strains. The information obtained from the API ZYM tests did not allow individual strains of *P. pentosaceus* to be discriminated from one another, but the authors suggested that the kit may be useful for discriminating *P. pentosaceus* from other organisms.

Table 5.6 Relative mobilities of the L-LDH and D-LDH enzymes of different pediococci*

Species	Relative mobility†	
	L-LDH	D-LDH
P. acidilactici	1.39	1.29
P. damnosus	0.92	1.16
P. dextrinicus	1.02‡	—§
P. halophilus	0.82	—
P. inopinatus	1.18	1.36
P. parvulus	0.97	1.42
P. pentosaceus	1.36	1.32
'*P. pentosaceus* subsp. *intermedius*'	1.38	1.23
P. urinae-equi	ND¶	ND

*Data from Back (1978a).
†Relative mobilities determined by acrylamide disc electrophoresis relative to rabbit heart L-LDH isoenzyme I.
‡Fructose 1,6-diphosphate-activated enzyme.
§—, activity not detected.
¶ND, not determined.

Schisler *et al.* (1979) identified brewery bacteria by analysing metabolic end-products by gas chromatography. They could differentiate *Pediococcus* spp. from other brewery bacteria but not between pediococci. Similar attempts to identify brewery microorganisms, including *Pediococcus* spp., were made by Hug and Bosio (1991), who employed high performance liquid chromatography to measure metabolic end-products.

Luchansky *et al.* (1992) successfully used clamped homogeneous electric field (CHEF) electrophoresis to discriminate strains of *P. acidilactici* used in sausage fermentation after low frequency-cleavage of chromosomal DNA by endonuclease *Asc*I.

5.12 Description of species which comprise the genus *Pediococcus*

The following information on the individual species of pediococci has been compiled mainly from the data of Sakaguchi (1958), Nakagawa and Kitahara (1959), Günther *et al.* (1962), Günther and White (1961a), Sakaguchi and Mori (1969), Dellaglio *et al.* (1974), Garvie (1974), Back (1978a,b), Dellaglio and Torriani (1986) and Tjandraatmadja *et al.* (1990) unless otherwise indicated. For synonyms, type strain details and priority see Table 5.1. For full details of the characteristics of each species, refer to Table 5.2.

5.12.1 Pediococcus acidilactici

The species name is derived from the Latin nouns, *acidium lacticum*, meaning lactic acid. Thus, *acidilactici* means 'of lactic acid'. Cells of *P. acidilactici* are 0.6–1 μm in diameter occurring singly, in pairs, tetrads or irregular clusters.

Growth occurs at pH 4.2 and 8.0 and sometimes at pH 8.5. Maximum temperature for growth is 50–53°C; all strains grow at 50°C. Optimum growth temperature is 40°C. Cells grow in the presence of 9–10% NaCl. The sugar fermentation reactions of *P. acidilactici* resemble those of *P. pentosaceus*. The inability of *P. acidilactici* to ferment maltose and its ability to grow at 50°C differentiate it from *P. pentosaceus*. An inability to hydrolyse starch and produce gas from gluconate separates the species from *P. dextrinicus*. An ability to hydrolyse arginine separates it from all other pediococci except for *P. pentosaceus*. Cells of this species are heat resistant. At 70°C, 10 min is needed to kill all cells in a culture. D and Z values have not been reported.

It is not always possible to differentiate between strains of *P. acidilactici* and *P. pentosaceus* on the basis of morphological, cultural and physiological traits alone. That this is so is highlighted by the fact that the type strain of *P. acidilactici* proposed by Garvie as the type species of the genus *Pediococcus* was later shown to belong to *P. pentosaceus* using DNA hybridization studies (Back and Stackebrandt, 1978). DL-Lactate is produced from glucose. Final pH in MRS broth is between 3.5 and 3.8. Mol% G+C is 38–44 (T_m).

Strains of *P. acidilactici* are widely distributed in fermenting plant material, including silage, cereal mashes and pickles, potato mashes and sake mashes, barley, malt, dried leaves and hay. Some have been isolated from salami. *Pediococcus acidilactici* has some application in semi-dry sausage production, miscellaneous fermentation processes and vitamin assays. Some strains produce pediocins.

5.12.2 Pediococcus damnosus

The species name is derived from the Latin adjective *damnosus*, which means 'destructive'. Cells of *P. damnosus* are 0.7–1.0 μm in diameter occurring singly, in pairs, tetrads or irregular clusters. Growth is slow, even on rich media.

Cells grow at pH 4.2 but not at pH 8.5. The maximum pH for growth is typically 6.5–7.0, optimum pH being in the range 4.0–6.0. Growth occurs in the range 8–30°C. At 25°C, growth is slow: 7–10 days are needed in some cases for colonies to reach their maximum size on MRS agar. Anaerobic incubation is essential for growth of most strains on agar media. Even at

6°C, most grow well in MRS after 3–5 weeks incubation. No growth occurs at 35°C, or above. Some strains grow in the presence of 4% NaCl. Few grow in the presence of 5% NaCl, but when they do, growth is slow and weak. None grow in the presence of 5.5% NaCl. The inability of *P. damnosus* to utilize starch and produce gas from gluconate allows it to be differentiated from *P. dextrinicus*. Its inability to hydrolyse arginine separates it from *P. acidilactici* and *P. pentosaceus*. Cells of *P. damnosus* are hop-tolerant. Exposure of some strains to hop bitter acids lead to formation of 'giant' cells, 5–15 μm in diameter (Nakagawa and Kitahara, 1962). Final pH in MRS broth is 3.7–4.2. Some strains form slime. DL-Lactate is produced from glucose. When galactose, sucrose or maltotriose are used as carbon source, the final pH in MRS broth is 4.8–5.0. Mol% G+C is 37–42 (T_m).

Pediococcus damnosus is associated with breweries and brewery products. It can be isolated from brewers' yeast, fermenting wort and beer, wine and cider (Back, 1978a). Cells of *P. damnosus* produces antibacterial substances which have a broad spectrum of activity, are not affected by proteolytic enzymes or catalase and are very heat resistant (Skyttä *et al.*, 1993). This property has not yet been exploited.

5.12.3 Pediococcus dextrinicus

The species name is derived from the Latin noun *dextrinosum*, which means dextrin. Thus, *dextrinicus* means 'relating to dextrin'. Cells are *c.* 1 μm diameter, occurring singly, in pairs, tetrads or irregular clusters. Occasionally short chains are formed.

Growth does not occur at pH 4.2, but does at pH 8.0. Maximum temperature for growth is 43–45°C. Optimum growth temperature is 32°C. Cells grow in the presence of 6% NaCl. In contrast to all other pediococci, *P. dextrinicus* utilizes starch and produces acid and gas from gluconate. The inability of *P. dextrinicus* to ferment pentoses allows the species to be differentiated from *P. acidilactici*, *P. pentosaceus*, *P. halophilus* and *P. urinae-equi*. Their inability to hydrolyse arginine separates them from *P. acidilactici* and *P. pentosaceus*. Uniquely among pediococci, *P. dextrinicus* possesses a fructose 1,6-diphosphate-activated L-LDH. L(+)-lactate is produced from glucose. *Pediococcus dextrinicus* is less anaerobic than other pediococci. 16S rRNA cataloguing has shown that the species is not closely related to other pediococci. Colonies develop on agar under aerobic conditions, but growth is improved by anaerobic incubation. Final pH in MRS broth is about 4.4. Mol% G+C is 40–41 (T_m).

Pediococcus dextrinicus has been isolated from silage, brewers' spent grains, beer and empty beer bottles. There are no applications for this organism at present.

5.12.4 Pediococcus halophilus

Note: Phylogenetic studies (Collins *et al.*, 1990) indicate that *P. halophilus* is more closely related to enterococci and carnobacteria than to other pediococci. Collins *et al.* (1990) propose that members of this species be transferred to a new genus '*Tetratogenococcus*' and named '*T. halophilus*'.

The species name currently used is derived from the Greek noun *halos*, which means 'salt', and the adjective *philus*, which means loving. These combine to give *halophilus*, which means salt-loving. Colonies of *P. halophilus* develop slowly on agar media under both aerobic and anaerobic conditions. Growth in broth is slow and less confluent than that seen with other *Pediococcus* spp.; 4–5 days is required for the cells to reach stationary phase.

Pediococcus halophilus is a heterogeneous species consisting of numerous biovars. Growth occurs at pH 9.0. Maximum temperature for growth is 37–40°C. Optimum temperature is 25–30°C: growth does not occur at 10°C or 45°C. Cells grow in the presence of 18% NaCl: some strains can grow with 20–26% NaCl. Growth of *P. halophilus* in MRS broth, and in other acidic media, is poor. Arginine is not usually hydrolysed, although strains have been isolated from pig faeces which are positive in this respect (Molitoris *et al.*, 1986). The salt-tolerance of *P. halophilus* allows members of this species to be easily separated from other pediococci. L(+)-Lactate is the major end-product of glucose metabolism. About 3% D(−)-lactate is also formed. Final pH in MRS broth is about 5.0. Mol% G+C is 34–36.5 (T_m).

Pediococcus halophilus can be isolated from soya sauce mashes, pickling brines, pickled anchovies, pig faeces and Tou-pan-chiang mash. Selected strains are used to inoculate fermented soya products and salted fermented foodstuffs.

5.12.5 Pediococcus inopinatus

The species name is derived from the Latin adjective *inopinatus*, which means 'unexpected'. Cells are 0.5–0.8 μm in diameter, occurring singly, in pairs, tetrads or irregular clusters. Growth of the organisms is slow and colonies on agar media take 5 days or more to reach their maximum size on MRS agar.

No growth occurs at pH 4.2. The maximum pH value for growth is 7.5. Maximum temperature for growth is 37–40°C. The optimum growth temperature lies between 30 and 32°C. Cells grow in the presence of 6–8% NaCl. *Pediococcus inopinatus* can be differentiated from *P. pentosaceus* and *P. acidilactici* by its inability to utilize pentoses and hydrolyse arginine. It differs from *P. dextrinicus* in being unable to grow on starch or produce acid and gas from gluconate. Unlike some strains of *P. damnosus*, it cannot utilize melezitose. An ability to utilize lactose separates *P. inopinatus* from *P. parvulus*. DL-Lactate is produced from glucose. Some strains form slime. The final pH in MRS broth is about 4.0. Mol% G+C is 39–40 (T_m).

Pediococcus inopinatus has been isolated from sauerkraut, beer yeast, hops, wine, empty beer bottles, fermented beans. There are no uses for this organism at present.

5.12.6 Pediococcus parvulus

The species name is derived from the Latin adjective *parvulus*, which means 'very small'. This name was originally chosen by Günther and White (1961a) because the organisms formed very small colonies on agar media. However, the size of the colonies can be increased by use of anaerobic incubation and by inclusion of Tween 80 in the growth medium. Cells are 0.7–1.1 μm in diameter occurring singly, in pairs, tetrads or irregular clusters.

Growth occurs at pH 4.5. The upper pH limit for growth lies between 7.0 and 7.5. Optimum pH for growth is about 6.5. The optimum growth temperature is about 30°C. Maximum temperature for growth is 37–39°C. Growth occurs in the presence of 5.5–8% NaCl. The inability of *P. parvulus* to utilize pentoses separates it from *P. pentosaceus* and *P. acidilactici*. Unlike *P. dextrinicus*, this species is unable to utilize starch or hydrolyse arginine. An inability to utilize lactose separates it from *P. inopinatus*. An inability to tolerate high NaCl concentrations separates the species from *P. halophilus*. DL-Lactate is formed from glucose. Some strains form slime. Final pH in MRS broth is 3.9–5.5 (the wide range is probably a reflection of the fact that some strains grow poorly). Mol% G+C is 40.5–41.6 (T_m).

Strains of *P. parvulus* have been isolated from sauerkraut, fermented vegetables, fermented beans, beer, cider and wine. Attempts have been made to use the organism to effect a malolactic fermentation of wine, with limited success.

5.12.7 Pediococcus pentosaceus

The species name is derived from the Latin noun *pentosum*, which means 'pentose'. Thus, *pentosaceus* means 'relating to a pentose'. Cells are 0.6–1.0 μm in diameter, occurring singly, in pairs, tetrads, or irregular clusters.

Growth occurs at pH 8.0 and pH 4.5. Maximum temperature for growth is 39–45°C. Cells of *P. pentosaceus* are less heat-resistant than those of *P. acidilactici*. Optimum growth temperature is 28–32°C. Cells grow in the presence of 9–10% NaCl. The sugar fermentation reactions of *P. pentosaceus* resemble those of *P. acidilactici*. The ability of *P. pentosaceus*

to ferment maltose and its lower growth temperature differentiate it from *P. acidilactici* (although not invariably). Its inability to hydrolyse starch and produce gas from gluconate separates it from *P. dextrinicus*. Lack of sucrose and melizitose fermentation separates this species from most other pediococci. Its ability to hydrolyse arginine separates it from all other pediococci with the exception of *P. acidilactici*.

Garvie (1986a) points out that, in some circumstances, strains of *P. pentosaceus* could be confused with micrococci since they form small colonies on sugar-free agar media, grow at pH 9.0 and, on media with low glucose content, may be weakly (pseudo-) catalase-positive. The rapid growth of *P. pentosaceus*, together with the low final pH which the organisms produce in broth media and their lack of cytochromes, serve to discriminate them from micrococci.

They produce DL-lactate from glucose. Final pH in MRS broth is between 3.5 and 3.8. Mol% G+C is 35–39 (T_m).

Pediococcus pentosaceus can be isolated from various plant materials including barley, malt, hops, dried leaves, hay, citrus fruits, apples and strawberries. The type strain (NCDO 990) was isolated from beer yeast. Selected strains of *P. pentosaceus* have been used to inoculate various fermentation processes including semi-dry sausage fermentations, cucumber and green bean fermentations, soya milk fermentations and silage. Some strains produce pediocins.

5.12.8 'Pediococcus pentosaceus *subsp.* intermedius'

Cells are 0.6–1 μm in diameter, occurring singly, in pairs, tetrads, or irregular clusters. Growth occurs at pH 4.5 and 8.0. Maximum temperature for growth is about 39°C. Most strains grow in the temperature range 8–45°C.

The sugar utilization reactions of '*P. pentosaceus* subsp. *intermedius*' are very similar to those of *P. pentosaceus*, except that arabinose, xylose and rhamnose are not fermented. Most strains give positive catalase reactions. DL-Lactate is formed from glucose. Final pH in MRS broth is between 3.5 and 3.8.

'*Pediococcus pentosaceus* subsp. *intermedius*' has been isolated from barley, malt, beer yeast, empty beer bottles, dried leaves, citrus fruits, strawberries, hay and other plant materials. There are no known uses for this subspecies.

5.12.9 Pediococcus urinae-equi

Note: The description of *P. urinae-equi* is given only for historical completeness. Strains fitting this description clearly belong to the genus *Aerococcus*.

The species name is derived from the Latin nouns *urina*, which means urine, and *equs*, which means horse. Thus, *urinae-equi* refers to horse urine. Cells are 0.8–1.0 μm in diameter, occurring singly, in pairs, tetrads or irregular clusters. On agar media, they form circular colonies, 1–2 mm in diameter, which are greyish-white in colour and raised. On agar stab cultures, growth occurs along the stab with limited surface growth.

The optimum pH value for growth is between 8.5 and 9.0. Growth will commence in media of initial pH 6.5–7.0. Final pH in broth is about 5.0. Temperature optimum is 25–30°C (max. 42°C). Cells produce L(+)-lactate from glucose. The electrophoretic behaviour of the LDH of this species has not been studied. Unlike other pediococci, growth of *P. urinae-equi* can take place in the absence of fermentable carbohydrate. Mol% G+C is 39.6–39.7 (T_m).

Reported isolations of *P. urinae-equi* are rare. Strains have been isolated from horse urine, rabbit dung and phak-gard-dong (Thai pickled vegetables) (Tanasupawat and Daengsubha, 1983). There are no known uses for this species.

5.13 Concluding remarks

On the basis of the evidence presently available, it seems appropriate to include the following species in the genus *Pediococcus*: *P. acidilactici*, *P. damnosus*, *P. dextrinicus*, *P. inopinatus*, *P. parvulus* and *P. pentosaceus*. The proposal of Back (1978a) that a subspecies of *P. pentosaceus* ('*P. pentosaceus* subsp. *intermedius*') be established, to accommodate strains of this species which are unable to utilize certain pentoses, also seems warranted.

Collins *et al.* (1990) have proposed that strains currently named *P. halophilus* be reclassified as '*Tetratogenococcus halophilus*' on the basis of 16S rRNA cataloguing of strain NCIMB 12011. It may be advisable to compare data from other strains of *P. halophilus* before adopting the suggestion, since *P. halophilus* is a heterogeneous species (Uchida, 1982).

The taxonomic position of strains belonging to *P. urinae-equi* is not in doubt. Both phenotypic and genotypic evidence points to their close association with the aerococci. These strains clearly do not belong within the genus *Pediococcus* and a review of their nomenclature would be appropriate.

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6 The genus *Lactococcus*

M. TEUBER

6.1 History

Lactococci are coccoid Gram-positive, anaerobic bacteria which produce L(+)-lactic acid from lactose in spontaneously fermented raw milk which is left at ambient temperatures around 20–30°C for 10–20 h. They are commonly called 'mesophilic lactic streptococci'. It is tempting to suggest that the first isolation, identification and description of the chemical entity lactic acid by Carl Wilhelm Scheele from sour milk in Sweden in the year 1780, was actually L(+)-lactic acid produced by lactococci. The microbial nature of lactic fermentation was recognized in 1857 by Louis Pasteur. The first bacterial pure culture on earth, obtained and scientifically described by Joseph Lister (1873) was *Lactococcus lactis*, at that time called: '*Bacterium lactis*'.

> Admitting then that we had here to deal with only one bacterium, it presents such peculiarities both morphologically and physiologically as to justify us, I think, in regarding it a definite and recognizable species for which I venture to suggest the name *Bacterium lactis*. This I do with diffidence, believing that up to this time no bacterium has been defined by reliable characters. Whether this is the only bacterium that can occasion the lactic acid fermentation, I am not prepared to say.

Around 1890, Storch in Copenhagen and Weigmann in Kiel isolated the mesophilic lactic streptococci responsible for the spontaneous fermentation of sour cream, sour milk and cheese and paved the way for their application as starter cultures for the dairy industry (for a detailed history, see von Milczewski, 1990). In 1909, Löhnis renamed '*Bacterium lactis*' as '*Streptococcus lactis*' mainly on the basis of the strains discovered in fermented dairy products. In the elegant taxonomy of lactic acid bacteria by Orla-Jensen (1919) the mesophilic lactic streptococci have a firm standing as '*Streptococcus lactis*' and '*Streptococcus cremoris*'. The serological differentiation scheme of the streptococci by Lancefield (1933) put the lactic streptococci into the group N which clearly separated them from the pathogenic streptococci (e.g. groups A, B, C) and enterococci (group D). Unfortunately, since 1993 the group N antiserum previously available from the Pasteur Institute (Paris) is no longer on the market.

The taxonomic confusion generated by the fact that quite unrelated

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